DESCRIPTION

PROLACTIN SECRETION MODULATOR

[Technical Field]

The present invention relates to an agent for modulating prolactin secretion and/or placental function, comprising a ligand polypeptide for a G protein-coupled receptor protein.

[Background Art]

Many hormones and neurotransmitters mediate biological functions through specific receptors present on the cell membrane. Many of these receptors engage themselves in the intracellular transduction of signals through activation of the coupled guanine nucleotide-binding protein (hereinafter sometimes referred to briefly as G protein) and have the common structure comprising 7 transmembrane domains. Therefore, these receptors are collectively referred to as G protein-coupled receptor or 7-transmembrane receptor.

One of the pathways to modulate biological functions mediated by such hormones or neurotransmitters through G protein-coupled receptors is the hypothalamo-pituitary system. Thus, the secretion of pituitary hormone from the hypophysis is controlled by hypothalamic hormones (pituitatropic releasing factor) and the functions of the target cells or organs are regulated through the pituitary hormones released into the circulation. This pathway carries out functional modulations of importance to the living body, such as homeostasis and regulation of the reproduction, development, metabolism and growth of individuals. The secretion of pituitary hormones is controlled by a positive or negative feedback mechanism involving hypothalamic hormone and the peripheral hormone secreted from the target endocrine gland. The

various receptor proteins present in the hypophysis are playing a central role in the regulation of the hypothalamus-pituitary system.

Meanwhile, it is known that these hormones and factors as well as their receptors are not localized in the hypothalamus-pituitary system but are broadly distributed in the brain. Therefore, it is suspected that, in the central nervous system, this substance called hypothalamus hormone is functioning as a neurotransmitter or a neuromodulator. Moreover, the substance is distributed in peripheral tissues as well and thought to be playing important roles in the respective tissue.

The pancreas is playing a crucial role in the carbohydrate metabolism by secreting glucagon and insulin as well as digestive juice. While insulin is secreted from the pancreatic β cells, its secretion is mainly stimulated by glucose. However, it is known that β cells have a variety of receptors and the secretion of insulin is controlled by a number of factors in addition to glucose as well as peptide hormones, e.g. galanine, somatostatin, gastric inhibitory polypeptide, glucagon, amyrin, etc.; sugars, e.g. mannose etc.; amino acids, and neurotransmitters, among others.

The means only heretofore available for identifying ligands for said G protein-coupled receptor proteins is estimation from the homology in primary structure of G protein-coupled receptor proteins.

Recently, investigation for novel opioid peptides by introducing a cDNA coding for a receptor protein to which a ligand is unknown, i.e. an orphan G protein-coupled receptor protein, into animal cells has been reported (Reinsheid, R. K. et al., Science, 270, 792-794, 1995, Menular, J.-C., et al., Nature 377, 532-535, 1995). However, in view of similarities to known G

protein-coupled receptor proteins and tissue distributions, it could be easily anticipated in these cases that the ligand would be belonging to the family of opioid peptides. The history of research and development in the realm of substances acting on the living body through the opioid receptor dates back to many years ago and various antagonists and agonists had been developed. Therefore, among the compounds artificially synthesized, an agonist of the receptor was picked out and, using it as a probe, expression of the receptor in the receptor cDNA-transfected cells was verified. Then, a search was made for an activator of the intracellular signal transduction which was similar to the agonist, the activator so found was purified, and the structure of the ligand was determined. However, when the homology of an orphan receptor to known G protein-coupled receptor proteins is low, it was very difficult to predict its ligand.

As Examples of the orphan G protein-coupled receptor, a human receptor protein (Genomics, vol.29, 335 (1995)) which is encoded by phGR3 (sometimes called GPR10) gene and a rat receptor protein, UHR-1 (Biochem. Biophy. Res. Commun., vol/209, 606 (1995)), is known.

Ligands for orphan G protein-coupled receptors expressed in the hypophysis, central nervous system, and pancreatic β cells are considered to be useful for developing medicines, but their structures and functions have not been elucidated as yet.

[Disclosure of Invension]

Employing a cell in which a cDNA coding for orphan G protein-coupled receptor protein, phGR3 has been expressed by a suitable means and using measurement of a specific cell stimulation activity exemplified by a signal transduction activity as an indicator, the inventors of the present invention succeeded in

screening a polypeptide derived from bovine, human, rat and determined their amino acid sequences and nucleotide sequences.

Furthermore, the inventors found that the ligand polypeptide has prolactin secretion and/or placental function(s).

The present invention, therefore, relates to

(1) an agent for modulating prolactin secretion which comprises a ligand polypeptide, or a salt thereof, for a G protein-coupled receptor protein,

(2) an agent as described in (1) above, wherein the ligand polypeptide is a polypeptide comprising an amino acid sequence represented by SEQ ID NO: 73 or a substantial equivalent thereto, or its amide or ester, or a salt thereof,

(3) an agent as described in (2) above, wherein the polypeptide comprising an amino acid sequence represented by SEQ ID NO: 73 is a polypeptide comprising an amino acid sequence represented by SEQ ID NO: 5, 8, 47, 50, 61 or 64,

(4) an agent as described in (1) above, which is for promoting prolactin secretion,

(5) an agent as described in (1) above, which is for inhibiting prolactin secretion,

(6) an agent as described in (4) above, which is for treating or preventing hypoovarianism, gonecyst cacogenesis, menopausal symdrome, or euthyroid hypometabolism,

(7) An agent as described in (5) above, which is for treating or preventing pituitary adenomatosis, brain tumor, emmeniopathy, autoimmune disease, prolactinoma, infertility, impotence, amenorrhea, galactorrhea, acromegaly, Chiari-Frommel symdrome, Argonz-del Castilo symdrome, Forbes-Albright symdrome, lymphoma, Sheehan syndrome or dyszoospermia,

(8) An agent for modulating placental function, which

comprises a ligand polypeptide, or a salt thereof, for a G protein-coupled receptor protein,

(9) An agent as described in (8) above, which is for treating or preventing choriocarcinomia, hydatid mole, irruption mole, abortion, unthrifty fetus, abnormal saccharometabolism, abnormal lipidmetabolism or oxytocia,

(10) An agent as described in (4) above, which is for promoting lactation of domestic mammal,

(11) An agent as described in (4) above, which is for an aphrodisiac,

(12) An agent for diagnosing function of prolactin secretion, which comprises a ligand polypeptide, or a salt thereof, for a G protein-coupled receptor protein,

(13) Use of a ligand polypeptide, or a salt thereof, for a G protein-coupled receptor protein for maufacture of a medicament for modulating prolactin secretion,

(14) A method for modulating prolactin secretion in a mammal, which comprises administering to said mammal a ligand polypeptide, or a salt thereof, for a G protein-coupled receptor protein,

(15) Use of a ligand polypeptide, or a salt thereof, for a G protein-coupled receptor protein for maufacture of a medicament for modulating placental function, and

(16) A method for modulating placental function in a mammal, which comprises administering to said mammal a ligand polypeptide, or a salt thereof, for a G protein-coupled receptor protein, and so on.

[Brief Description of the Drawings]

Fig. 1 shows the nucleotide sequence of the human pituitary-derived G protein-coupled receptor protein cDNA fragment harbored in cDNA clone p19P2 isolated by PCR using human pituitary-derived cDNA and the amino

acid encoded by the nucleotide sequence. The primer used for sequencing was -21M13. The underscored region corresponds to the synthetic primer.

Fig. 2 shows the nucleotide sequence of the human pituitary-derived G protein-coupled receptor protein cDNA fragment harbored in cDNA clone p19P2 isolated by PCR using human pituitary-derived cDNA and the amino acid sequence encoded thereby. The primer used for sequencing was M13RV-N (Takara). The underscored region corresponds to the synthetic primer.

Fig. 3 shows a partial hydrophobic plot of the protein encoded by the human pituitary-derived G protein-coupled receptor protein cDNA fragment harbored in p19P2 constructed according to the amino acid sequence shown in Fig. 1.

Fig. 4 shows a partial hydrophobic plot of the protein encoded by the human pituitary-derived G protein-coupled receptor protein cDNA fragment harbored in p19P2 constructed according to the amino acid sequence shown in Fig. 2.

Fig. 5 is a diagram comparing the partial amino acid sequence of the protein encoded by the human pituitary-derived G protein-coupled receptor protein cDNA fragment harbored in p19P2 as shown in Figs. 1 and 2 with the known G protein-coupled receptor protein S12863. The shadowed region represents the region of agreement. The 1 to 9 amino acid sequence of p19P2 corresponds to the 1 to 99 amino acid sequence of Fig. 1 and the 156 to 230 amino acid sequence corresponds to the 1 to 68 amino acid sequence of Fig. 2.

Fig. 6 shows the nucleotide sequence of the MIN6-derived G protein-coupled receptor protein cDNA fragment based on the nucleotide sequences of the MIN6-derived G protein-coupled receptor protein cDNA fragments harbored in the cDNA clones pG3-2 and pG1-10 isolated by PCR using MIN6-derived cDNA and the amino

acid sequence encoded by the nucleotide sequence. The underscored region corresponds to the synthetic primer.

Fig. 7 is a diagram comparing the partial amino acid sequence encoded by pG3-2/pG1-10 of the MIN6-derived G protein-coupled receptor protein shown in Fig. 6 with the partial amino acid sequence of the protein encoded by p19P2 shown in Figs. 1 and 2. The shadowed region corresponds to the region of agreement. The 1 to 99 amino acid sequence of the protein encoded by p19P2 corresponds to the 1 to 99 amino acid sequence of Fig. 1 and the 156 to 223 amino acid sequence corresponds to the 1 to 68 amino acid sequence of Fig. 2. The 1 to 223 amino acid sequence of the protein encoded by pG3-2/pG1-10 corresponds to the 1 to 223 amino acid sequence of Fig. 6.

Fig. 8 is a partial hydrophobic plot of the MIN6-derived G protein-coupled receptor protein constructed according to the partial amino acid sequence shown in Fig. 6. Fig. 9 shows the entire nucleotide sequence of the human pituitary-derived G protein-coupled receptor protein cDNA harbored in the cDNA clone phGR3 isolated from a human pituitary-derived cDNA library by the plaque hybridization method using the DNA fragment inserted in p19P2 as a probe and the amino acid sequence encorded by the nucleotide sequence.

Fig. 10 shows the result of Northern blotting of human pituitary mRNA hybridized with radioisotope-labeled human pituitary cDNA clone phGR3.

Fig. 11 shows a hydrophobic plot of the protein encoded by the human pituitary-derived G protein-coupled receptor protein cDNA harbored in the phGR3 as constructed according to the amino acid sequence shown in Fig. 9.

Fig. 12 shows the nucleotide sequence of the MIN6-derived G protein-coupled receptor protein cDNA fragment harbored in the cDNA clone p5S38 isolated by

PCR using MIN6-derived cDNA and the amino acid sequence encoded by the nucleotide sequence. The underscored region corresponds to the synthetic primer.

Fig. 13 shows a diagram comparing the partial amino acid sequence of MIN6-derived G protein-coupled receptor protein encoded by p5S38 shown in Fig. 12 with the partial amino acid sequence of G protein-coupled receptor protein encoded by the cDNA fragment harbored in p19P2 as shown in Figs. 1 and 2 and the partial amino acid sequence of G protein-coupled receptor protein encoded by the nucleotide sequence generated from the nucleotide sequences of cDNA fragments contained in pG3-2 and pG1-10 shown in Fig. 6. The shadowed region represents the sequence region of agreement. The 1 to 144 amino acid sequence of the protein encoded by p5S38 corresponds to the 1 to 144 amino acid sequence of Fig. 12, the 1 to 99 amino acid sequence of the protein encoded by p19P2 corresponds to the 1 to 99 amino acid sequence of Fig. 1 and the 156 to 223 amino acid sequence corresponds to 1 to 68 amino acid sequence of Fig. 2. The 1 to 223 amino acid sequence of the protein encoded by pG3-2/pG1-10 corresponds to the 1 to 223 amino acid sequence of Fig. 6.

Fig. 14 shows a partial hydrophobic plot of the protein encoded by the MIN6-derived G protein-coupled receptor protein cDNA harbored in p5S38 as constructed according to the partial amino acid sequence shown in Fig. 12.

Fig. 15 shows the results of the following analysis. Thus, RT-PCR was carried out to confirm the expression of mRNA in CHO cells transfected by pAKKO-19P2. Lanes 1-7 represent the results obtained by performing PCRs using serial dilutions of pAKKO-19P2 for comparison, i.e. the 10μl/ml stock solution (lane 1), 1/2 dilution (lane 2), 1/4 dilution (lane 3), 1/64 dilution (Lane 4),

1/256 dilution (lane 5), 1/1024 dilution (lane 6), and 1/4096 dilution (lane 7) of the plasmid as templates, and analyzing the reaction mixtures by 1.2% agarose gel electrophoresis. Lanes 8 through 11 are the results obtained by performing PCRs using a 1/10 dilution (lane 8), a 1/100 dilution (lane 9), and a 1/1000 dilution (lane 10) of the cDNA prepared from the CHO-19P2 cell line as templates and subjecting the respective reaction mixtures to electrophoresis. Lane 11 was obtained by performing PCR using a template obtained by carrying out cDNA synthesis without reverse transcriptase and subjecting the PCR reaction product to electrophoresis. Lanes 12 and 13 were obtained by performing PCR using cDNAs prepared from mock CHO cells with and without addition of reverse transcriptase, respectively, as templates and subjecting the respective reaction products to electrophoresis. M represents the DNA size marker. The lanes at both ends were obtained by electrophoresing 1 μ l of λ /Sty I digest (Nippon Gene) and the second lane from right was obtained with 1 μ l of ϕ X 174/Hinc II digest (Nippon Gene). The arrowmark indicates the position of the band amplified by PCR of about 400 bp.

Fig. 16 shows the activity of the crude ligand peptide fraction extracted from rat whole brain to promote release of arachidonic acid metabolites from CHO-19P2 cells. The arachidonic acid metabolite releasing activity was expressed as % of the amount of [^{3}H] arachidonic acid metabolites released in the presence of the crude ligand polypeptide fraction with the amount of [^{3}H] arachidonic acid metabolites released in the presence of 0.05% BAS-HABB being taken as 100%. The activity to promote release of arachidonic acid metabolites from the CHO-19P2 cell line was detected in a 30% CH_3CN fraction.

Fig. 17 shows the activity of the crude ligand

polypeptide fraction extracted from bovine hypothalamus to promote release of arachidonic acid metabolites from CHO-19P2 cells. The arachidonic acid metabolite release-promoting activity was expressed as % of the amount of [^{3}H] arachidonic acid metabolites released in the presence of the crude ligand polypeptide fraction with the amount of [^{3}H] arachidonic acid metabolites released in the presence of 0.05% BAS-HABB being taken as 100%. The activity to promote release of arachidonic acid metabolites from the CHO-19P2 cell line was detected in a 30% CH_3CN fraction just as in the crude ligand polypeptide fraction from rat whole brain.

Fig. 18 shows the activity of the fraction purified with the reversed-phase column C18 218TP5415 to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells. The active fraction from RESOURCE S was fractionated on C18 218TP5415. Thus, chromatography was carried out at a flow rate of 1 ml/min. on a concentration gradient of 20%-30% CH_3CN /0.1% TFA/H_2O, the eluate was collected in 1 ml fractions, and each fraction was lyophilized. Then, the activity of each fraction to specifically promote release of arachidonic acid metabolites from the CHO-19P2 cell line was determined. As a result, the activity was fractionated into 3 fractions (designated, in the order of elution, as P-1, P-2, and P-3).

Fig. 19 shows the activity of the fraction purified with the reversed-phase column diphenyl 219TP5415 to specifically promote arachidonic acid metabolite release from CHO-19P2 cells. The P-3 active fraction from C18 218TP5415 was fractionated on diphenyl 219TP5415. The chromatography was carried out at a flow rate of 1 ml/min. on a concentration gradient of 22%-25% CH_3CN /0.1% TFA/ H_2O, the eluate was collected in 1 ml fractions, and each fraction was lyophilized.

Then, the activity to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells in each fraction was determined. As a result, the activity converged in a single peak.

Fig. 20 shows the activity of the fraction purified by reversed-phase column μ RPC C2/C18 SC 2.1/10 to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells. The peak active fraction from diphenyl 219TP5415 was fractionated on μ RPC C2/C18 SC 2.1/10. The chromatography was carried out at a flow rate of 100 μ l/min. on a concentration gradient of 22%-23.5% CH_3CN /0.1% TFA/ H_2O, the eluate was collected in 100 μ l fractions, and each fraction was lyophilized. Then, the activity to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells in each fraction was determined. As a result, the activity was found as two peaks of apparently a single substance (peptide).

Fig. 21 shows the activity of the P-2 fraction purified by reversed-phase column þRPC C2/C18 SC 2.1/10 to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells. The chromatography was carried out at a flow rate of 100 þl/min. on a concentration gradient of 21.5%-23.0% CH_3CN /0.1% TFA/distilled H_2O, the eluate was collected in 100 μ l fractions, and each fraction was lyophilized. Then, the activity to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells in each fraction was determined. As a result, the activity was found as a peak of apparently a single substance.

Fig. 22 shows the nucleotide sequence of bovine hypothalamus ligand polypeptide cDNA fragment as derived from the nucleotide sequence of the bovine hypothalamus-derived ligand polypeptide cDNA fragment which specifically promotes release of arachidonic acid

metabolites from CHO-19P2 cells as harbored in a cDNA clone isolated by PCR using bovine hypothalamus-derived cDNA and the amino acid sequence encoded by said nucleotide sequence. The region indicated by the arrowmark corresponds to the synthetic primer.

Fig. 23 shows the nucleotide sequence of the bovine hypothalamus-derived ligand polypeptide cDNA fragment generated according to the nucleotide sequence of the bovine hypothalamus-derived ligand polypeptide cDNA fragment which specifically promotes release of arachidonic acid metabolites from CHO-19P2 cells as harbored in a cDNA clone isolated by PCR using bovine hypothalamus-derived cDNA and the amino acid sequence encoded by said nucleotide sequence. The region indicated by the arrowmark corresponds to the synthetic primer.

Fig. 24 shows the amino acid sequences (a) and (b) of the bovine hypothalamus-derived ligand polypeptides which specifically promote release of arachidonic acid metabolites from CHO-19P2 cells and the cDNA sequence coding for the full coding region of the ligand polypeptides defined by SEQ ID NO:1 and SEQ ID NO:44.

Fig. 25 shows the concentration-dependent activity of synthetic ligand polypeptide (19P2-L31) to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells. The synthetic peptide was dissolved in degassed distilled H_2O at a final concentration of 10^{-3}M and diluted with 0.05% BSA-HBSS to concentrations of 10^{-12}M-10^{-6}M. The arachidonic acid metabolite releasing activity was expressed in the measured radioactivity of [^{3}H] arachidonic acid metabolites released in the supernatant when the dilution was added to the cells. As a result, the activity of 19P2-31 to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells was found in a concentration-dependent manner.

Fig. 26 shows the concentration-dependent activity of synthetic ligand polypeptide (19P2-L31(O)) to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells. The synthetic ligand peptide was dissolved in degassed distilled H_2O at a final concentration of 10^{-3}M and diluted with 0.05% BSA-HBSS to concentrations of 10^{-12}M-10^{-6}M. The arachidonic acid metabolite releasing activity was expressed in the measured radioactivity of [^{3}H] arachidonic acid metabolites released in the supernatant when the dilution was added to the cells. As a result, the activity of 19P2-L31(O) to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells was found in a dose-dependent manner.

Fig. 27 shows the activity of synthetic ligand polypeptide 19P2-L20 to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells. The synthetic peptide was dissolved in degassed distilled H_2O at a final concentration of 10^{-3}M and diluted with 0.05% BSA-HBSS to concentrations of 10^{-12}M-10^{-6}M. The arachidonic acid metabolite releasing activity was expressed in the measured radioactivity of [^{3}H] arachidonic acid metabolites released in the supernatant when the dilution was added to the cells. As a result, the activity of 19P2-L20 to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells was found in a dose-dependent manner.

Fig. 28 shows the 1.2% agarose gel electrophoregram of the DNA fragments of the phages cloned from a bovine genomic library as digested with restriction enzymes BamHI(B) and SalI(S). As the DNA size marker (M), StyI digests of λ phage DNA were used. In lane B, two bands derived from the vector were detected in positions between the first (19,329 bp) and second (7.743 bp) marker bands, as well as 3 bands derived from the inserted fragment between the third (6,223 bp) and 5th

(3,472 bp) bands. In lane S, two bands derived from the vector were similarly detected but due to the overlap of the band of the inserted fragment, the upper band is thicker than the band in lane B.

Fig. 29 shows the nucleotide sequence around the coding region as decoded from bovine genomic DNA. The 1st to 3rd bases (ATG) correspond to the translation start codon and the 767th to 769th bases (TAA) correspond to the translation end codon.

Fig. 30 shows a comparison between the nucleotide sequence (genome) around the coding region as deduced from bovine genomic DNA and the nucleotide sequence (cDNA) of bovine cDNA cloned by PCR. The sequence region of agreement is indicated by shading. As to the 101st to 572nd region, there is no corresponding region in the nucleotide sequence of cDNA, indicating that it is an intron.

Fig. 31 shows the translation of the amino acid sequence encoded after elimination of the intron from the nucleotide sequence around the coding region as decoded from bovine genomic DNA.

Fig. 32 shows the full-length amino acid sequence and the cDNA sequence coding for the full coding region of rat ligand polypeptide.

Fig. 33 shows amino acid sequence of bovine ligand polypeptide and the nucleotide sequences of DNAs coding for bovine polypeptide and rat polypeptide. The arrowmark indicates the region corresponding to the synthetic primer.

Fig. 34 shows the full-length amino acid sequence and the sequence of cDNA coding for the full coding region of human ligand polypeptide.

Fig. 35 shows a comparison of the amino acid sequences in the translation region of bovine ligand polypeptide, rat ligand polypeptide, and human ligand polypeptide.

Fig. 36 shows the results of a receptor binding experiment with an iodine-labeled ligand polypeptide in living cells.

Fig. 37 shows the arachidonic acid metabolite releasing activity of the ligand polypeptide in CHO-19P2-9 and CHO-UHR1.

Fig. 38 shows the results of RT-PCR assays of UHR-1 expressed in rat tissues. Each value is the mean ± S.E.M. of 3 experiments.

Fig. 39 shows the results of RT-PCR assays of the ligand polypeptide expressed in rat tissues. Each value is the mean ± S.E.M. of 3 experiments.

Fig. 40 shows the influence of the ligand polypeptide on the glucose-induced plasma insulin concentration determined by radioimmunoassay.

Fig. 41 shows the measured motor activity of mice treated with 10 nmol of the ligand polypeptide. (a): spontaneous motor activity, (b): rearing

Fig. 42 shows the measured motor activity of mice treated with 1 nmol of the ligand polypeptide. (a): spontaneous motor activity, (b): rearing

Fig. 43 shows the measured motor activity of mice treated with 0.1 nmol of the ligand polypeptide. (a): spontaneous motor activity, (b): rearing

Fig. 44 shows the measured motor activity of mice treated with 0.01 nmol of the ligand polypeptide. (a): spontaneous motor activity, (b): rearing

Fig. 45 shows the change in the body temperature of mice upon administration of the ligand polypeptide into the cerebral ventricle 15 hours following subcutaneous administration of 3 mg/kg reserpine. The single asterisk * stands for $p<0.05$ and the double asterisk ** for $p<0.01$.

Fig. 46 shows a schematic diagram showing a microinjection cannula inserted into the area postrema (AP) at an angle of 20 degrees.

Fig. 47 shows a typical example of pulse wave and mean blood pressure following injection of the ligand polypeptide into AP [Conscious rat, 10 nmol at a flow rate of 1 μl/min].

Fig. 48 shows the plasma GH level following administration of the ligand polypeptide 50 nmol into the third ventricle of rats under pentobarbital anesthesia.

Fig. 49 shows the plasma GH level following administration of the ligand polypeptide into the third ventricle.

To unrestrained conscious rats, the ligand polypeptide or PBS was administered into the third ventricle following intraatrial injection of GHRH 5 μg/kg. The point of time at which the polypeptide was administered was reckoned as 0 min. *: $p<0.05$; **: $p<0.01$.

Fig. 50 shows the relationship of ligand polypeptide antiserum with absorbance.

Fig. 51 shows the results of determination of arachidonic acid metabolite releasing activity of the anti-ligand polypeptide polyclonal antibody.

Fig. 52 shows the nucleotide sequence of the full coding region of rat UHR-1 constructed on the expression vector pAKKO-UHR-1 and the amino acid sequence encoded thereby.

Fig. 53 shows the nucleotide sequence of the inserted fragment of plasmid pmGB3. → indicates the sequence corresponding to the primer.

Fig. 54 shows the predicted cDNA and translated protein based on the nucleotide sequence of plasmid pmGB3. → indicates the sequence corresponding to the primer. The sequence flanked by ↓ ↓ is the sequence predicted to be an intron.

Fig. 55 shows the change in prolactin release from rat pituitary RC-4B/C cells upon addition of

ligand polypeptide 19P2-L31.

Fig. 56 shows the change in prolactin secretion from primary cultured rat pituitary cells upon addition of ligand polypeptide 19P2-L31.

Fig. 57 shows the time course of expression of UHR-1 gene in the rat placenta described in Example 48.

Fig. 58 shows the time course of plasma prolactin concentration after administration of 19P2-L31 in unrestrained male rats. *=$p<0.05$. Each value is the mean ± S.E.M. of 3-4 experiments.

Fig. 59 shows the time course of plasma prolactin concentration after administration of 19P2-L31 in unrestrained female rats. *=$p<0.05$. Each value is the mean ± S.E.M. of 3-4 experiments.

Fig. 60 shows the time course of plasma prolactin concentration was determined among the sexual cycle.

[Best Mode for Carrying Out the Invention]

In the specification and drawings of the present application, the abbreviations used for bases (nucleotides), amino acids and so forth are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature or those conventionally used in the art. Examples thereof are given below. Amino acids for which optical isomerism is possible are, unless otherwise specified, in the L form.

DNA : Deoxyribonucleic acid
cDNA : Complementary deoxyribonucleic acid
A : Adenine
T : Thymine
G : Guanine
C : Cytosine
RNA : Ribonucleic acid
mRNA : Messenger ribonucleic acid
dATP : Deoxyadenosine triphosphate
dTTP : Deoxythymidine triphosphate

dGTP : Deoxyguanosine triphosphate
dCTP : Deoxycytidine triphosphate
ATP : Adenosine triphosphate
EDTA : Ethylenediamine tetraacetic acid
SDS : Sodium dodecyl sulfate
EIA : Enzyme Immunoassay
G, Gly: Glycine (or Glycyl)
A, Ala: Alanine (or Alanyl)
V, Val: Valine (or Valyl)
L, Leu: Leucine (or Leucyl)
I, Ile: Isoleucine (or Isoleucyl)
S, Ser: Serine (or Seryl)
T, Thr: Threonine (or Threonyl)
C, Cys: Cysteine (or Cysteinyl)
M, Met: Methionine (or Methionyl)
E, Glu: Glutamic acid (or Glutamyl)
D, Asp: Aspartic acid (or Aspartyl)
K, Lys: Lysine (or Lysyl)
R, Arg: Arginine (or Arginyl)
H, His: Histidine (or Histidyl)
F, Phe: Phenylalamine (or Phenylalanyl)
Y, Tyr: Tyrossine (or Tyrosyl)
W, Trp: Tryptophan (or Tryptophanyl)
P, Pro: Proline (or Prolyl)
N, Asn: Asparagine (or Asparaginyl)
Q, Gln: Glutamine (or Glutaminyl)
pGlu: Pyroglutamic acid (or Pyroglutamyl)
Me: Methyl
Et: Ethyl
Bu: Butyl
Ph: Phenyl
TC: Thiazolidinyl-4(R)-carboxamide

In this specification, substitutions, protective groups and reagents commonly used are indicated by the following abbreviations:

BHA : benzhydrylamine

pMBHA : p-methylbenzhydrylamine
Tos : p-toluenesulfonyl
CHO : formyl
HONB : N-hydroxy-5-norbornene-2,3-dicarboxyimide
OcHex : cyclohexyl ester
Bzl : benzyl
Cl_2-Bzl : dichloro-benzyl
Bom : benzyloxymethyl
Br-Z : 2-bromobenzyloxycarbonyl
Boc : t-butoxycarbonyl
DCM : dichloromethane
HOBt : 1-hydroxybenztriazole
DCC : N,N'-dicyclohexylcarbodiimide
TFA : trifluoro acetate
DIEA : diisopropylethylamine
Fmoc : N-9-fluorenylmethoxycarbonyl
DNP : dinitrophenyl
Bum : t-butoxymethyl
Trt : trityl

As used herein the term "substantial equivalent(s)" means that the activity of the protein, e.g., nature of the binding activity of the ligand and the receptor and physical characteristics are substantially the same. Substitutions, deletions or insertions of amino acids often do not produce radical changes in the physical and chemical characteristics of a polypeptide, in which case polypeptides containing the substitution, deletion, or insertion would be considered to be substantially equivalent to polypeptides lacking the substitution, deletion, or insertion.

Substantially equivalent substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. (1) The non-polar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. (2) The

polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. (3) The positively charged (basic) amino acids include arginine, lysine and histidine. (4) The negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

The amino acids being comprised the ligand polypeptide of the present invention may form D-form or L-form, but usually form L-form.

The ligand polypeptide according to the present invention is a polypeptide which is capable of binding to G protein-coupled receptor protein and comprising an amino acid sequence represented by SEQ ID NO:73 or its substantial equivalent thereto, or its amide or ester, or a salt thereof(hereinafter sometimes referred to briefly as the ligand polypeptide or the polypeptide).

In SEQ ID NO:73, Xaa at 10th position is Ala or Thr; Xaa at 11th position is Gly or Ser; and Xaa at 21th position is H, Gly, or GlyArg.

Preferable example of the amino acid sequence represented by SEQ ID NO:73 is the amino acid sequence represented by SEQ ID NO:5, 8, 47, 50, 61 or 64. Among them, the amino acid sequence represented by SEQ ID NO:61 or 64 is more preferable. Further, the amino acid sequence represented by SEQ ID 64 is more preferable.

The above ligand polypeptide of the present invention includes any polypeptides derived from any tissues, e.g. pituitary gland, pancreas, brain, kidney, liver, gonad, thyroid gland, gall bladder, bone marrow, adrenal gland, skin, muscle, lung, digestive canal, blood vessel, heart, etc.; or cells of man and other warm-blooded animals, e.g. guinea pig, rat, mouse, swine, sheep, bovine, monkey, etc. and comprising an amino acid sequence represented by SEQ ID NO:73, preferably the amino acid sequence represented by SEQ ID NO:5, 8, 47, 50, 61 or 64, or its substantial

equivalent thereto. For example, in addition to the protein comprising the amino acid sequence of SEQ ID NO:73, preferably the amino acid sequence represented by SEQ ID NO:5, 8, 47, 50, 61 or 64, the ligand polypeptide of the present invention includes the protein comprising an amino acid sequence having a homology of about 50-99.9%, preferably 70-99.9%, more preferably 80-99.9% and especially preferably 90-99.9% to the amino acid sequence of SEQ ID NO:73, preferably the amino acid sequence represented by SEQ ID NO:5, 8, 47, 50, 61 or 64, and having qualitatively substantially equivalent activity to the protein comprising the amino acid sequence of SEQ ID NO:73, preferably the amino acid sequence represented by SEQ ID NO:5, 8, 47, 50, 61 or 64. The term "substantially equivalent" means the nature of the receptor-binding activity, signal transduction activity and the like is equivalent. Thus, it is allowable that even differences among grades such as the strength of receptor binding activity and the molecular weight of the polypeptide are present.

To be more specific, the ligand polypeptide of the present invention includes the polypeptide derived from the rat whole brain, bovine hypothalamus, or human whole brain and comprising the amino acid sequence of SEQ ID NO:73. In addition, the ligand polypeptide of the present invention includes the polypeptides which comprises substantially equivalent polypeptides such as (1) polypeptides wherein 1 to 15, preferably 1 to 10, and more preferably 1 to 5 amino acid residues are deleted from the amino acid sequence of SEQ ID NO:73, (2) polypeptides wherein 1 to 80, preferably 1 to 50, more preferably 1 to 10 amino acid residues are added to the amino acid sequence of SEQ ID NO:73, (3) polypeptides wherein 1 to 15, preferably 1 to 10, more preferably 1 to 5 amino acid residues are

substituted with one or more other amino acid residues of the amino acid sequence of SEQ ID NO:73, or

(4) polypeptide wherein the amino acid, especially its side chain, of the polypeptide of the above (1) to (3) is modified, or its amide thereof, or its ester thereof, or a salt thereof.

Among them, preferred is the polypeptide comprising the amino acid sequence of SEQ ID NO:73 and the polypeptide comprising the amino acid sequence which a peptide of SEQ ID NO:74 is added to the N-terminus of the polypeptide comprising the amino acid sequence of SEQ ID NO:73.

The ligand polypeptide of the present invention can be changed or mutated by substitution, deletion, addition or modification as mentioned above (1) to (4), to a polypeptide which is stable against heat or proteases, or a polypeptide whose physiological function is activated.

The ligand polypeptide or an amide thereof, or an ester thereof, or a salt thereof includes the changed or mutated polypeptide mentioned above.

The peptides described in this specification, the left ends are the N-terminus (amino terminus) and the right end is the C-terminus (carboxyl terminus) according to the convention of the peptide indication.

Furthermore, the polypeptide or partial peptide of the present invention includes those wherein the N-terminal side of Gln is cleaved in vivo to form pyroglutamyl peptide.

While the C-terminus of the polypeptide of the present invention, for example the polypeptide comprising the amino acid sequence of SEQ ID NO:73, is usually carboxyl (-COOH) or carboxylate (-COO-), it may be amide ($-CONH_2$) or ester (-COOR) form. The ester residue R includes a C_{1-6} alkyl group such as methyl, ethyl, n-propyl, isopropyl, n-butyl, etc., a C_{3-8}

cycloalkyl group such as cyclopentyl, cyclohexyl, etc., a C_{6-12} aryl group such as phenyl, α-naphthyl, etc., and a C_{7-14} aralkyl group such as a phenyl-C_{1-2} alkyl group, e.g. benzyl, phenethyl, benzhydryl, etc. or an α-naphthyl-C_{1-2} alkyl, e.g. α-naphthylmethyl etc. In addition, the ester may be a pivaloyloxymethyl ester which is broadly used for oral administration.

When the polypeptide of the present invention, for example the polypeptide comprises the amino acid sequence of SEQ ID NO:73, has a carboxyl or carboxylate group in any position other than the C-terminus, the corresponding amide or ester are also included in the concept of the polypeptide of the present invention. The ester mentioned just above includes the esters mentioned for the C-terminus.

The preferred ligand polypeptide of the present invention is a peptide which the C-terminus is amidated. Especially preferred is a polypeptide comprising the amino acid sequence of SEQ ID NO:5, 8, 47, 50, 61 or 64 which the C-terminus is amidated.

The salt of polypeptide of the present invention includes salts with physiologically acceptable bases, e.g. alkali metals or acids such as organic or inorganic acids, and is preferably a physiologically acceptable acid addition salt. Examples of such salts are salts thereof with inorganic acids, e.g. hydrochloric acid, phosphoric acid, hydrobromic acid or sulfuric acid, etc. and salts thereof with organic acids, e.g. acetic acid, formic acid, propionic acid, fumaric acid, maleic acid, succinic acid, tartaric acid, citric acid, malic acid, oxalic acid, benzoic acid, methanesulfonic acid or benzenesulfonic acid, etc.

The ligand polypeptide or amide or ester, or a salt thereof of the present invention may be

(1) manufactured from the tissues or cells of warm-blooded animals inclusive of human by purifying

techniques or

(2) manufactured by the peptide synthesis as described hereinafter.

(3) Moreover, it can be manufactured by culturing a transformant carrying a DNA coding for the polypeptide as described hereinafter.

(1) In the production from the tissues or cells of human or other warm-blooded animals, the ligand polypeptide can be purified and isolated by a process which comprises homogenizing the tissue or cells of human or other warm-blooded animal, extracting the homogenate with an acid, for instance, and subjecting the extract to a combination of chromatographic procedures such as reversed-phase chromatography, ion-exchange chromatography, affinity chromatography, etc.

(2) As mentioned above, the ligand polypeptide in the present invention can be produced by the per se known procedures for peptide synthesis. The methods for peptide synthesis may be any of a solid-phase synthesis and a liquid-phase synthesis. Thus, the objective peptide can be produced by condensing a partial peptide or amino acid capable of constituting the protein with the residual part thereof and, when the product has a protective group, the protective group is detached whereupon a desired peptide can be manufactured. The known methods for condensation and deprotection includes the procedures described in the following literature (1)-(5).

(1) M. Bodanszky and M. A. Ondetti, Peptide Synthesis, Interscience Publishers, New York, 1966

(2) Schroeder and Luebke, The Peptide, Academic Press, New York, 1965

(3) Nobuo Izumiya et al., Fundamentals and Experiments in Peptide Synthesis, Maruzen, 1975

(4) Haruaki Yajima and Shumpei Sakakibara, Biochemical Experiment Series 1, Protein Chemistry IV,

205, 1977

(5) Haruaki Yajima (ed.), Development of Drugs-Continued, 14, Peptide Synthesis, Hirokawa Shoten

After the reaction, the protein can be purified and isolated by a combination of conventional purification techniques such as solvent extraction, column chromatography, liquid chromatography, and recrystallization. Where the protein isolated as above is a free compound, it can be converted to a suitable salt by the known method. Conversely where the isolated product is a salt, it can be converted to the free peptide by the known method.

The amide of polypeptide can be obtained by using a resin for peptide synthesis which is suited for amidation. The resin includes chloromethyl resin, hydroxymethyl resin, benzhydrylamine resin, aminomethyl resin, 4-benzyloxybenzyl alcohol resin, 4-methylbenzhydrylamine resin, PAM resin, 4-hydroxymethylmethylphenylacetamidomethyl resin, polyacrylamide resin, 4-(2',4'-dimethoxyphenyl-hydroxymethyl)phenoxy resin, 4-(2',4'-dimethoxyphenyl-Fmoc aminoethyl)phenoxy resin, and so on. Using such a resin, amino acids whose α- amino groups and functional groups of side-chain have been suitably protected are condensed on the resin according to the sequence of the objective peptide by various condensation techniques which are known per se. At the end of the series of reactions, the peptide or the protected peptide is removed from the resin and the protective groups are removed to obtain the objective polypeptide.

For the condensation of the above-mentioned protected amino acids, a variety of activating reagents for peptide synthesis can be used but a carbodiimide compound is particularly suitable. The carbodiimide includes DCC, N,N'-diisopropylcarbodiimide, and N-ethyl-N'-(3-dimethylaminoprolyl)carbodiimide. For

activation with such a reagent, a racemization inhibitor additive, e.g. HOBt and the protected amino acid are directly added to the resin or the protected amino acid pre-activated as symmetric acid anhydride, HOBt ester, or HOOBt ester is added to the resin. The solvent for the activation of protected amino acids or condensation with the resin can be properly selected from among those solvents which are known to be useful for peptide condensation reactions. For example, N,N-dimethylformamide, N-methylpyrrolidone, chloroform, trifluoroethanol, dimethyl sulfoxide, DMF, pyridine, dioxane, methylene chloride, tetrahydrofuran, acetonitrile, ethyl acetate, or suitable mixtures of them can be mentioned. The reaction temperature can be selected from the range hitherto-known to be useful for peptide bond formation and is usually selected from the range of about -20℃ - 50℃. The activated amino acid derivative is generally used in a proportion of 1.5-4 fold excess. If the condensation is found to be insufficient by a test utilizing the ninhydrin reaction, the condensation reaction can be repeated to achieve a sufficient condensation without removing the protective group. If repeated condensation still fails to provide a sufficient degree of condensation, the unreacted amino group can be acetylated with acetic anhydride or acetylimidazole.

The protecting group of amino group for the starting material amino acid includes Z, Boc, tertiary-amyloxycarbonyl, isobornyloxycarbonyl, 4-methoxybenzyloxycarbonyl, Cl-Z, Br-Z, adamantyloxycarbonyl, trifluoroacetyl, phthalyl, formyl, 2-nitrophenylsulfenyl, diphenylphosphinothioyl, or Fmoc. The carboxy-protecting group that can be used includes but is not limited to the above-mentioned C_{1-6} alkyl, C_{3-8} cycloalkyl and C_{7-14} aralkyl as well as 2-adamantyl, 4-nitrobenzyl, 4-methoxybenzyl, 4-chlorobenzyl, phenacyl,

benzyloxycarbonylhydrazido, tertiary-butoxycarbonylhydrazido, and tritylhydrazido.

The hydroxy group of serine and threonine can be protected by esterification or etherification. The group suited for said esterification includes carbon-derived groups such as lower alkanoyl groups, e.g. acetyl etc., aroyl groups, e.g. benzoyl etc., benzyloxycarbonyl, and ethoxycarbonyl. The group suited for said etherification includes benzyl, tetrahydropyranyl, and tertiary-butyl.

The protective group for the phenolic hydroxyl group of tyrosine includes Bzl, C_{12}-Bzl, 2-nitrobenzyl, Br-Z, and tertiary-butyl.

The protecting group of imidazole for histidine includes Tos, 4-methoxy-2,3,6-trimethylbenzenesulfonyl, DNP, benzyloxymethyl, Bum, Boc, Trt, and Fmoc.

The activated carboxyl group of the starting amino acid includes the corresponding acid anhydride, azide, and active esters, e.g. esters with alcohols such as pentachlorophenol, 2,4,5-trichlorophenol, 2,4-dinitrophenol, cyanomethyl alcohol, p-nitrophenol, HONB, N-hydroxysuccinimide, N-hydroxyphthalimide, HOBt, etc. The activated amino group of the starting amino acid includes the corresponding phosphoramide.

The method for elimination of protective groups includes catalytic reduction using hydrogen gas in the presence of a catalyst such as palladium black or palladium-on-carbon, acid treatment with anhydrous hydrogen fluoride, methanesulfonic acid, trifluoromethanesulfonic acid, trifluoroacetic acid, or a mixture of such acids, base treatment with diisopropylethylamine, triethylamine, piperidine, piperazine, reduction with sodium metal in liquid ammonia. The elimination reaction by the above-mentioned acid treatment is generally carried out at a temperature of -20 ℃ - 40 ℃ and can be conducted

advantageously with addition of a cation acceptor such as anisole, phenol, thioanisole, m-cresol, p-cresol, dimethyl sulfide, 1,4-butanedithiol, 1,2-ethanedithiol. The 2,4-dinitrophenyl group used for protecting the imidazole group of histidine can be eliminated by treatment with thiophenol, while the formyl group used for protecting the indole group of tryptophan can be eliminated by alkali treatment with dilute sodium hydroxide solution or dilute aqueous ammonia as well as the above-mentioned acid treatment in the presence of 1,2-ethanedithiol, 1,4-butanedithiol.

The method for protecting functional groups which should not take part in the reaction of the starting material, the protective groups that can be used, the method of removing the protective groups, and the method of activating the functional groups that are to take part in the reaction can all be selected from among the known groups and methods.

An another method for obtaining the amide form of the polypeptide comprises amidating the α - carboxyl group of the C-terminal amino acid at first, then extending the peptide chain to the N-side until the desired chain length, and then selectively deprotecting the α - amino group of the C-terminal peptide and the α - carboxy group of the amino acid or peptide that is to form the remainder of the objective polypeptide and condensing the two fragments whose - amino group and side-chain functional groups have been protected with suitable protective groups mentioned above in a mixed solvent such as that mentioned hereinbefore. The parameters of this condensation reaction can be the same as described hereinbefore. From the protected peptide obtained by condensation, all the protective groups are removed by the above-described method to thereby provide the desired crude peptide. This crude peptide can be purified by known purification

procedures and the main fraction be lyophilized to provide the objective amidated polypeptide.

To obtain an ester of the polypeptide, the α-carboxyl group of the C-terminal amino acid is condensed with a desired alcohol to give an amino acid ester and then, the procedure described above for production of the amide is followed.

The ligand polypeptide of the present invention, its amide or ester, or a salt thereof can be any peptide that has the same activities, e.g. pituitary function modulating activity, central nervous system function modulating activity, pancreatic function modulating activity, prolactin secretion modulating activity or placental function modulating activity, as the polypeptide which has an amino acid sequence of SEQ ID NO:73 or its substantial equivalent thereto. As such peptides, there can be mentioned peptides wherein 1 to 15 amino acids residues are deleted from the above-mentioned amino acid sequence of SEQ ID NO:73. To be specific, the peptide having an amino acid sequence corresponding to the 2nd to 21st positions of the amino acid sequence of SEQ ID NO:73, the peptide corresponding to the 3rd to 21st positions of the amino acid sequence of SEQ ID NO:73, the peptide corresponding to the 4th to 21st positions of the amino acid sequence of SEQ ID NO:73, the peptide corresponding to the 5th to 21st positions of the amino acid sequence of SEQ ID NO:73, the peptide corresponding to the 6th to 21st positions of the amino acid sequence of SEQ ID NO:73, the peptide corresponding to the 7th to 21st positions of the amino acid sequence of SEQ ID NO:73, the peptide corresponding to the 8th to 21st positions of the amino acid sequence of SEQ ID NO:73, the peptide corresponding to the 9th to 21st positions of the amino acid sequence of SEQ ID NO:73, the peptide

corresponding to the 10th to 21st positions of the amino acid sequence of SEQ ID NO:73, the peptide corresponding to the 11th to 21st positions of the amino acid sequence of SEQ ID NO:73, the peptide corresponding to the 12th to 21st positions of the amino acid sequence of SEQ ID NO:73, the peptide corresponding to the 13th to 21st positions of the amino acid sequence of SEQ ID NO:73, the peptide corresponding to the 14th to 21st positions of the amino acid sequence of SEQ ID NO:73, and the peptide corresponding to the 15th to 21st positions of the amino acid sequence of SEQ ID NO:73, can be mentioned as preferred examples. Moreover, the peptide having the amino acid sequence of SEQ ID NO:74 is also preferred.

Examples of the ligand polypeptide for the polypeptide comprises the amino acid sequence of SEQ ID NO:5, 8, 47, 50 or 61 each of which is an preferable example of the polypeptide comprising the amino acid sequence of SEQ ID NO:73, are the same as the cases of the polypeptide comprising the amino acid sequence of SEQ ID NO:73, mentioned above.

The DNA coding for the ligand polypeptide or a partial peptide thereof of the present invention may be any DNA comprising the nucleotide sequence encoding a polypeptide having an amino acid sequence of SEQ ID NO:73 or its substantial equivalent thereto. Furthermore, the DNA may be any of genomic DNA, genomic DNA library, tissue- or cell-derived cDNA, tissue- or cell-derived cDNA library, and synthetic DNA. The vector for such as library may be any of bacteriophage, plasmide, cosmide, and phagimide. Moreover, it can be directly amplified by the RT-PCR(reverse transcription PCR) method by using an RNA fraction may be prepared from a tissue or cells .

To be more specific, as the DNA coding for a

polypeptide derived from rat whole brain or bovine hypothalamus and comprising the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:44, the DNA comprising the nucleotide sequence of SEQ ID NO:2 can be exemplified. In SEQ ID NO:2, R at 129th position represents G or A, and Y at 179th and 240th positions represents C or T. When Y at 179th position is C, the amino acid sequence of SEQ ID NO:1 is encoded, and when Y at 179th position is T, the amino acid sequence of SEQ ID NO:44 is encoded.

As the DNA coding for a bovine-derived polypeptide comprising the amino acid sequence of SEQ ID NO:3, 4, 5, 6, 7, 8, 9 or 10, a DNA comprising the nucleotide sequence of SEQ ID NO:11, 12, 13, 14, 15, 16, 17 or 18 can be exemplified. Here, R at 63th position of SEQ ID NO:11, 13, 14 or 15 and R at 29th position of SEQ ID NO:12, 16, 17, or 18 represent G or A.

As the DNA coding for a rat-derived polypeptide of SEQ ID NO:45, 47, 48, 49, 50, 51, or 52, a DNA comprising the nucleotide sequence of SEQ ID NO:46, 53, 54, 55, 56, 57, or 58 can be exemplified.

Furthermore, as the DNA coding for a human-derived peptide of SEQ ID NO:59, 61, 62, 63, 64, 65, or 66, a DNA comprising the nucleotide sequence of SEQ ID NO:60, 67, 68, 69, 70, 71, or 72 can be exemplified.

Among DNAs coding for the bovine-derived polypeptide comprising the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:44, the rat-derived polypeptide comprising the amino acid sequence of SEQ ID NO:45, or the human-derived polypeptide comprising the amino acid sequence of SEQ ID NO:59, DNA fragments comprising partial nucleotide sequences of 6 to 90, preferably 6 to 60, more preferably 9 to 30, and especially preferably 12 to 30 can be advantageously used as DNA probes as well.

The DNA coding for the ligand polypeptide or a

partial peptide thereof of the present invention can be produced by the following genetic engineering procedures.

(3) The DNA fully encoding the polypeptide of the present invention can be cloned either by PCR amplification using synthetic DNA primers having a partial nucleotide sequence of the polypeptide or partial peptide or by hybridization using the DNA inserted in a suitable vector and labeled with a DNA fragment comprising a part or full region of a human-derived polypeptide or a synthetic DNA. The hybridization can be carried out typically by the procedure described in Molecular Cloning (2nd ed., J. Sambrook et al., Cold Spring Harbor Lab. Press, 1989). When a commercial library is used, the instructions given in the accompanying manual can be followed.

The cloned DNA coding for the polypeptide or partial peptide can be used directly or after digestion with a restriction enzyme or addition of a linker depending on purposes. This DNA has ATG as the translation initiation codon at the 5' end and may have TAA, TGA, or TAG as the termination codon at the 3' end. The translation initiation and termination codons can be added by means of suitable DNA adapters.

An expression vector for the polypeptide or partial peptide can be produced by, for example (a) cutting out a target DNA fragment from the DNA for the polypeptide or partial peptide of the present invention and (b) ligating the target DNA fragment with the downstream side of a promoter in a suitable expression vector.

The vector may include plasmids derived from Escherichia coli, e.g., pBR322, pBR325, pUC12, pUC13, etc.; plasmids derived from Bacillus subtilis, e.g., pUB110, pTP5, pC194, etc.; plasmids derived from yeasts e.g., pSH19, pSH15, etc.; bacteriophages such as λ-phage, and animal virus such as retrovirus, vaccinia

virus and baculovirus.

According to the present invention, any promoter can be used as long as it is compatible with the host cell which is used for expressing a gene. When the host for the transformation is E. coli, the promoters are preferably trp promoters, lac promoters, recA promoters, λPL promoters, lpp promoters, etc. When the host for the transformation is Bacillus, the promoters are preferably SPO1 promoters, SPO2 promoters, penP promoters, etc. When the host is a yeast, the promoters are preferably PHO5 promoters, PGK promoters, GAP promoters, ADH promoters, etc. When the host is an animal cell, the promoters include SV40-derived promoters, retrovirus promoters, metallothionein promoters, heat shock promoters, cytomegalovirus (CMV) promoters, SR α promoters, etc. An enhancer can be effectively utilized for expression.

As required, furthermore, a host-compatible signal sequence is added to the N-terminal side of the polypeptide or partial peptide thereof. When the host is E. coli, the utilizable signal sequences may include alkaline phosphatase signal sequences, OmpA signal sequences, etc. When the host is Bacillus, they may include α-amylase signal sequences, subtilisin signal sequences, etc. When the host is a yeast, they may include mating factor α signal sequences, invertase signal sequences, etc. When the host is an animal cell, they may include insulin signal sequences, α-interferon signal sequences, antibody molecule signal sequences, etc.

A transformant or transfectant is produced by using the vector thus constructed, which carries the polypeptide or partial peptide-encoding DNA of the present invention. The host may be, for example, Escherichia microorganisms, Bacillus microorganisms, yeasts, insect cells, animal cells, etc. Examples of

the Escherichia include Escherichia coli K12.DH1 [Proc. Natl. Acad. Sci. USA, Vol. 60, 160 (1968)], JM103 [Nucleic Acids Research, Vol. 9, 309 (1981)], JA221 [Journal of Molecular Biology, Vol. 120, 517 (1978)], HB101 [Journal of molecular Biology, Vol, 41, 459 (1969)], C600 [Genetics, Vol. 39, 440 (1954)], etc. Examples of the Bacillus microorganism are, for example Bacillus subtilis MI114 [Gene, Vol. 24, 255 (1983)], 207-21 [Journal of Biochemistry, Vol. 95, 76 (1984)], etc. The yeast may be, for example, Saccharomyces cerevisiae AH22, AH22R$^-$, NA87-11A, DKD-5D, 20B-12, etc. The insect may include a silkworm (Bombyx mori larva), [Maeda et al, Nature, Vol. 315, 592 (1985)] etc. The host animal cell may be, for example, monkey-derived cell line, COS-7, Vero, Chinese hamster ovary cell line (CHO cell), DHFR gene-deficient Chinese hamster cell line (dhfr$^-$ CHO cell), mouse L cell, mouse myeloma cell, human FL, etc.

Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. Transformation of Escherichia microorganisms can be carried out in accordance with methods as disclosed in, for example, Proc. Natl. Acad. Sci. USA, Vol. 69, 2110 (1972), Gene, Vol. 17, 107 (1982), etc. Transformation of Bacillus microorganisms can be carried out in accordance with methods as disclosed in, for example, Molecular & General Genetics, Vol. 168, 111 (1979), etc. Transformation of the yeast can be carried out in accordance with methods as disclosed in, for example, Proc. Natl. Acad. Sci. USA, Vol. 75, 1929 (1978), etc. The insect cells can be transformed in accordance with methods as disclosed in, for example, Bio/Technology, 6, 47-55, 1988. The animal cells can be transformed by methods as disclosed in, for example, Virology, Vol. 52, 456, 1973, etc. The transformants or transfectants harboring the expression vector

carrying a polypeptide or partial peptide thereof encoding DNA are produced according to the aforementioned techniques.

Cultivation of the transformant (transfectant) in which the host is Escherichia or Bacillus microorganism can be carried out suitably in a liquid culture medium. The culture medium may contains carbon sources, nitrogen sources, minerals, etc. necessary for growing the transformant. The carbon source may include glucose, dextrin, soluble starch, sucrose, etc. The nitrogen source may include organic or inorganic substances such as ammonium salts, nitrates, corn steep liquor, peptone, casein, meat extracts, bean-cakes, potato extracts, etc. Examples of the minerals may include calcium chloride, sodium dihydrogen phosphate, magnesium chloride, etc. It is further allowable to add yeasts, vitamines, growth-promoting factors, etc. It is desired that the culture medium is pH from about 5 to about 8.

The culture medium for Escherichia microorganism is preferably an M9 medium containing, for example, glucose and casamino acids (Miller, Journal of Experiments in Molecular Genetics), 431-433, Cold Spring Harbor Laboratory, New York, 1972. Depending on necessity, the medium may be supplemented with drugs such as 3β-indolyl acrylic acid in order to improve efficiency of the promoter. In the case of an Escherichia host, the cultivation is carried out usually at about 15 to 43℃ for about 3 to 24 hours. As required, aeration and stirring may be applied. In the case of Bacillus host, the cultivation is carried out usually at about 30 to 40℃ for about 6 to 24 hours. As required, aeration and stirring may be also applied. In the case of the transformant in which the host is a yeast, the culture medium used may include, for example, a Burkholder minimum medium [Bostian, K.L. et al., Proc.

Natl. Acad. Sci. USA, Vol. 77, 4505 (1980)], an SD medium containing 0.5% casamino acids [Bitter, G.A. et al., Proc. Natl. Acad. Sci. USA, Vol. 81, 5330 (1984)], etc. It is preferable that the pH of the culture medium is adjusted to be from about 5 to about 8. The cultivation is carried out usually at about 20 to 35℃ for about 24 to 72 hours. As required, aeration and stirring may be applied. In the case of the transformant in which the host is an insect, the culture medium used may include those obtained by suitably adding additives such as passivated (or immobilized) 10% bovine serum and the like to the Grace's insect medium (Grace, T.C.C., Nature, 195, 788 (1962)). It is preferable that the pH of the culture medium is adjusted to be about 6.2 to 6.4. The cultivation is usually carried out at about 27℃ for about 3 to 5 days. As desired, aeration and stirring may be applied. In the case of the transformant in which the host is an animal cell, the culture medium used may include MEM medium [Science, Vol. 122, 501 (1952)], DMEM medium [Virology, Vol. 8, 396 (1959)], RPMI 1640 medium [Journal of the American Medical Association, Vol. 199, 519 (1967)], 199 medium [Proceedings of the Society of the Biological Medicine, Vol. 73, 1 (1950)], etc. which are containing, for example, about 5 to 20% of fetal calf serum. It is preferable that the pH is from about 6 to about 8. The cultivation is usually carried out at about 30 to 40℃ for about 15 to 60 hours. As required, medium exchange, aeration and stirring may be applied.

Separation and purification of the polypeptide from the above-mentioned cultures can be carried out according to methods described herein below.

To extract polypeptide from the cultured microorganisms or cells, the microorganisms or cells are collected by known methods after the cultivation,

suspended in a suitable buffer solution, disrupted by ultrasonic waves, lysozyme and/or freezing and thawing, etc. and, then, a crude extract of the polypeptide or partial peptide is obtained by centrifugation or filtration. Other conventional extracting or isolating methods can be applied. The buffer solution may contain a protein-denaturing agent such as urea or guanidine hydrochloride or a surfactant such as Triton X-100 (registered trademark, hereinafter often referred to as "TM").

In the case where the polypeptide is secreted into culture medium, supernatant liquid is separated from the microorganisms or cells after the cultivation is finished and the resulting supernatant liquid is collected by widely known methods. The culture supernatant liquid and extract containing the polypeptide or partial peptide can be purified by a suitable combination of widely known methods for separation, isolation and purification. The widely known methods of separation, isolation and purification may include methods which utilizes solubility, such as salting out or sedimentation with solvents methods which utilizes primarily a difference in the molecular size or weight, such as dialysis, ultrafiltration, gel filtration and SDS-polyacrylamide gel electrophoresis, methods utilizing a difference in the electric charge, such as ion-exchange chromatography, methods utilizing specific affinity such as affinity chromatography, methods utilizing a difference in the hydrophobic property, such as reverse-phase high-performance liquid chromatography, and methods utilizing a difference in the isoelectric point such as isoelectric electrophoresis, or chromatofocusing, etc.

In cases where the polypeptide thus obtained is in a free form, the free protein can be converted into a salt thereof by known methods or method analogous

thereto. In case where the polypeptide thus obtained is in a salt form vice versa, the protein salt can be converted into a free form or into any other salt thereof by known methods or method analogous thereto.

The polypeptide produced by the transformant can be arbitrarily modified or a polypeptide can be partly removed therefrom, by the action of a suitable protein-modifying enzyme before or after the purification. The protein-modifying enzyme may include trypsin, chymotrypsin, arginyl endopeptidase, protein kinase, glycosidase, etc. The activity of the polypeptide thus formed can be measured by experimenting the coupling (or binding) with receptor or by enzyme immunoassays (enzyme linked immunoassays) using specific antibodies.

The ligand polypeptide of the present invention has prolactin secretion modulating activity, i.e. prolactin secretion promoting and/or inhibiting activities. Thus, as will be understood from the Examples presented hereinafter, the ligand polypeptide of the present invention has prolactin secretion promoting activity and, therefore, finds application as a drug for preventing and/or treating various diseases associated with prolactin hyposecretion. On the other hand, the ligand polypeptide of the invention has a high affinity for the receptor proteins and, therefore, when used in an increased dose, causes desensitization for prolactin secretion, thus exhibiting prolactin secretion inhibiting activity. In this sense, it can be used as a drug for preventing and/or treating various diseases associated with prolactin hypersecretion.

Therefore, the ligand polypeptide of the invention can be used with advantage as a prolactin secretion-stimulating agent for the prevention and treatment of certain diseases associated with prolactin secretion, such as hypoovarianism, gonecyst cacogenesis,

menopausal symdrome, euthyroid hypometabolism. In addition, the ligand polypeptide of the invention can be used with advantage as a aphrodisiac.

On the other hand, the ligand polypeptide of the invention can be used with advantage as a prolactin secretion inhibitory agent in the prevention and treatment of certain diseases associated with prolactin secretion, such as pituitary adenomatosis, brain tumor, emmeniopathy, autoimmune disease, prolactinoma, infertility, impotence, amenorrhea, galactorrhea, acromegaly, Chiari-Frommel symdrome, Argonz-del Castilo symdrome, Forbes-Albright symdrome, lymphoma, Sheehan syndrome or dyszoospermia.

In addition, the ligand poltpeptide of the present invention is used as a contraceptives based on its prolactin secretion inhibitory activity.

In addition, the ligand polypeptide of the invention can be used as a test reagent for study of the prolactin secretory function or a veterinary drug for use as a lactogogue in mammalian farm animals such as bovine, goat, and swine, and is even expected to find application in the elaboration of useful substances in such farm mammals and harvesting of the substances secreted into their milk.

In addition, the ligand polypeptide of the present invention has a function of modulating placental function, and can be used as an agent for treating or preventing chriocarcinomia, hydatid mole, irruption mole, abortion, unthrifty fetus, abnormal saccharometabolism, abnormal lipidmetabolism or oxytocia.

When the ligand polypeptide of the present invention is used as a pharmaceutical composition as described above, it can be used by conventional methods. For example, it can be used orally in the form of tablets which may be sugar coated as necessary,

capsules, elixirs, microcapsules etc., or non-orally in the form of injectable preparations such as aseptic solutions and suspensions in water or other pharmaceutically acceptable liquids. These preparations can be produced by mixing the polypeptide with physiologically acceptable carriers, flavoring agents, excipients, vehicles, antiseptics, stabilizers, binders etc. in unit dosage forms required for generally accepted manners of pharmaceutical making. Active ingredient contents in these preparations are set so that an appropriate dose within the specified range is obtained.

Additives which can be mixed in tablets, capsules etc. include binders such as gelatin, corn starch, tragacanth and gum arabic, excipients such as crystalline cellulose, swelling agents such as corn starch, gelatin and alginic acid, lubricants such as magnesium stearate, sweetening agents such as sucrose, lactose and saccharin, and flavoring agents such as peppermint, akamono oil and cherry. When the unit dosage form is the capsule, the above-mentioned materials may further incorporate liquid carriers such as oils and fats. Sterile compositions for injection can be formulated by ordinary methods of pharmaceutical making such as by dissolving or suspending active ingredients, naturally occuring vegetable oils such as sesame oil and coconut oil, etc. in vehicles such as water for injection.

Aqueous liquids for injection include physiological saline and isotonic solutions containing glucose and other auxiliary agents, e.g., D-sorbitol, D-mannitol and sodium chloride, and may be used in combination with appropriate dissolution aids such as alcohols, e.g., ethanol, polyalcohols, e.g., propylene glycol and polyethylene glycol, nonionic surfactants, e.g., polysorbate 80 (TM) and HCO-50 etc. Oily liquids

include sesame oil and soybean oil, and may be used in combination with dissolution aids such as benzyl benzoate and benzyl alcohol. Furthermore the above-mentioned materials may also be formulated with buffers, e.g., phosphate buffer and sodium acetate buffer; soothing agents, e.g., benzalkonium chloride, procaine hydrochloride; stabilizers, e.g., human serum albumin, polyethylene glycol; preservatives, e.g., benzyl alcohol, phenol; antioxidants etc. Normally, an appropriate ampule is filled in with the thus prepared injectable liquid. Because the thus-obtained preparation is safe and of low toxicity, it can be administered to humans or warm-blooded mammals, e.g., mouse, rats, guinea pig, rabbits, chicken, sheep, pigs, bovines, cats, dogs, monkeys, baboons, chimpanzees, for instance.

The dose of said polypeptide is normally about 0.1-100 mg, preferably 1.0-50 mg, and more preferably 1.0-20 mg per day for a patient of euthyroid hypometabolism (weighing 60 kg) in oral administration, depending on symptoms etc. In non-oral administration, it is advantageous to administer the polypeptide in the form of injectable preparation at a daily dose of about 0.01-30 mg, preferably about 0.1-20 mg, and more preferably about 0.1-10 mg per administration by an intravenous injection for a patient of euthyroid hypometabolism (weighing 60 kg), depending on subject of administration, target organ, symptoms, method of administration etc. For other animal species, corresponding does as converted per 60 kg weight can be administered.

The G protein-coupled receptor protein for the above ligand polypeptide of the present invention may be any of G protein-coupled receptor proteins derived from various tissues, e.g. hypophysis, pancreas, brain, kidney, liver, gonad, thyroid gland, gall bladder, bone

marrow, adrenal gland, skin, muscle, lung, alimentary canal, blood vessel, heart, etc. of human and other warm-blooded animals, e.g. guinea pig, rat, mouse, swine, sheep, bovine, monkey, etc.; and comprising an amino acid sequence of SEQ ID NO:19, 20, 21, 22 or 23, or a substantial equivalent thereto. Thus, the G protein-coupled receptor protein includes, in addition to proteins comprising the SEQ ID NO:19, 20, 21, 22 or 23, those proteins comprising amino acid sequences of about 90-99.9% homology to the amino acid sequence of SEQ ID NO:19, 20, 21, 22 or 23 and having qualitatively substantially equivalent activity to proteins comprising the amino acid sequence of SEQ ID NO:19, 20, 21, 22, or 23. The activities which these proteins possess may include ligand binding activity and signal transduction activity. The term "substantially equivalent" means that the nature of the ligand binding activity and the like is equivalent. Therefore, it is allowable that even differences among grades such as the strength of ligand binding activity and the molecular weight of receptor protein are present.

To be further specific, the G protein-coupled receptor proteins include human pituitary-derived G protein-coupled receptor proteins which comprises the amino acid sequence of SEQ ID NO:19 or/and SEQ ID NO:20, mouse pancreas-derived G protein-coupled receptor proteins which comprises the amino acid sequence of SEQ ID NO:22, and mouse pancreas-derived G protein-coupled receptor proteins which comprises the amino acid sequence of SEQ ID NO:23. As the human pituitary-derived G protein-coupled receptor proteins which comprises the amino acid sequence of SEQ ID NO:19 and/or SEQ ID NO:20 include the human pituitary-derived G protein-coupled receptor protein which comprises the amino acid sequence of SEQ ID NO:21. The G protein-coupled receptor proteins further include proteins

wherein 1 to 30 amino acid residues, preferably 1 to 10 amino acid residues are deleted from the amino acid sequence of SEQ ID NO:19, 20, 21, 22 or 23, proteins wherein 1 to 30 amino acid residues, preferably 1 to 10 amino acid residues are added to the amino acid sequence of SEQ ID NO:19, 20, 21, 22, or 23, the proteins wherein 1 to 30 amino acid residues, preferably 1 to 10 amino acid residues in the amino acid sequence of SEQ ID NO:19, 20, 21, 22, or 23 are substituted with one or more other amino acid residues.

Here, the protein which comprises an amino acid sequence of SEQ ID NO:21 or a substantial equivalent thereto contains the full-length of the amino acid sequence for human pituitary-derived G protein-coupled receptor protein. The protein which comprises an amino acid sequence of SEQ ID NO:19 or/and SEQ ID NO:20 or a substantial equivalent thereto may be a partial peptide of the protein which comprises an amino acid sequence of SEQ ID NO:21 or a substantial equivalent thereto. The protein which comprises an amino acid sequence of SEQ ID NO:22 or SEQ ID NO:23 or a substantial equivalent thereto is a G protein-coupled receptor protein which is derived from mouse pancreas but since its amino acid sequence is quite similar to the amino acid sequence of SEQ ID NO:19 or/and SEQ ID NO:20 (cf. Example 8, Fig. 13 in particular), the protein which comprises an amino acid sequence of SEQ ID NO:22 or 23 or a substantial equivalent thereto is also subsumed in the category of said partial peptide of the protein which comprises an amino acid sequence of SEQ ID NO:21 or a substantial equivalent thereto.

Thus, the above-mentioned protein comprising an amino acid sequence of SEQ ID NO:21 or a substantial equivalent thereto or a partial peptide of the protein or a salt thereof, which will be described below, includes the protein comprising an amino acid sequence

of SEQ ID NO:19, 20, 22, or 23 or a substantial equivalent thereto, or a salt thereof.

Furthermore, the G protein-coupled receptor protein includes the protein in which the N-terminal Met has been protected with a protective group, e.g. C_{1-6} acyl such as formyl or acetyl, the protein in which the N-terminal side of Gln has been cleaved in vivo to form pyroglutamic acid, the protein in which the side chain of any relevant constituent amino acid has been protected with a suitable protective group, e.g. C_{1-6} acyl such as formyl or acetyl, and the complex protein such as glycoproteins available upon attachment of sugar chains.

The salt of G protein-coupled receptor protein includes the same kinds of salts as mentioned for the ligand polypeptide.

The G protein-coupled receptor protein or a salt thereof or a partial peptide thereof can be produced from the tissues or cells of human or other warm-blooded animals by the per se known purification technology or, as described above, by culturing a transformant carrying a DNA coding for the G protein-coupled receptor protein. It can also be produced in accordance with the procedures for peptide synthesis which are described above. The procedures for peptide synthesis is described in WO96/05302 in detail.

A partial peptide of G protein-coupled receptor protein may include, for example, a fragment containing an extracellular portion of the G protein-coupled receptor protein, i.e. the site which is exposed outside the cell membranes. Examples of the partial peptide are fragments containing a region which is an extracellular area (hydrophilic region) as analyzed in a hydrophobic plotting analysis of the G protein-coupled receptor protein, such as shown in Fig. 3, Fig. 4, Fig. 8, Fig. 11, or Fig. 14. Furthermore, a

fragment which partly contains a hydrophobic region may also be used. While peptides which separately contains each domain may be used too, peptides which contains multiple domains at the same time will be used as well.

The salt of a partial peptide of G protein-coupled receptor protein may be the same one as mentioned for the salt of ligand polypeptide.

The DNA coding for the G protein-coupled receptor protein may be any DNA comprising a nucleotide sequence encoding the G protein-coupled receptor protein which comprises an amino acid sequence of SEQ ID NO:19, 20, 21, 22, or 23 or a substantial equivalent thereto. It may also be any one of genomic DNA, genomic DNA library, tissue- or cell-derived cDNA, tissue- or cell-derived cDNA library, and synthetic DNA. The vector for such a library may include bacteriophage, plasmid, cosmid, and phargimide. Furthermore, using an RNA fraction prepared from a tissue or cells, a direct amplification can be carried out by the RT-PCR method.

To be specific, the DNA encoding the human pituitary-derived G protein-coupled receptor protein which comprises the amino acid sequence of SEQ ID NO:19 include a DNA which comprises the nucleotide sequence of SEQ ID NO:24. The DNA encoding the human pituitary-derived G protein-coupled receptor protein which comprises the amino acid sequence of SEQ ID NO:20 include a DNA which comprises the nucleotide sequence of SEQ ID NO:25. The DNA encoding the human pituitary-derived G protein-coupled receptor protein which comprises the amino acid sequence of SEQ ID NO:21 include a DNA which comprises the nucleotide sequence of SEQ ID NO:26. The DNA encoding the mouse pancreas-derived G protein-coupled receptor protein which comprises the amino acid sequence of SEQ ID NO:22 include a DNA which comprises the nucleotide sequence of SEQ ID NO:27. The DNA encoding the mouse pancreas-

derived G protein-coupled receptor protein which comprises the amino acid sequence of SEQ ID NO:23 include a DNA comprising the nucleotide sequence of SEQ ID NO:28.

A method for cloning the DNA completely coding for the G protein-coupled receptor protein, vector, promoter, host cell, a method for transformation, a method for culturing the transformant or a method for separation and purification of the G protein-coupled receptor protein may include the same one as mentioned for the ligand polypeptide.

To be specific, the plasmid phGR3 obtained in Example 5, described hereinafter, is digested with the restriction enzyme SalI and the translation frame for the full-length cDNA encoding hGR3 is isolated. This frame is subjected to ligation to, for example, the expression vector pAKKO-111 for animal cell use which has been treated with BAP (bacterial alkaline phosphatase) after SalI digestion for inhibition of autocyclization. After completion of the ligation reaction, a portion of the reaction mixture is used for transfection of, for example, Escherichia coli DH5. Among the transformants obtained, a transformant in which the cDNA coding for hGR3 has been inserted in the forward direction with respect to a promoter, such as SR α, which has been inserted into the expression vector beforehand is selected by mapping after cleavage with restriction enzymes or by nucleotide sequencing and the plasmid DNA is prepared on a production scale.

The thus-constructed DNA of the expression vector is introduced into CHO dhfr$^-$ cells using a kit for introducing a gene into animal cells by the calcium phosphate method, the liposome method or the like to provide a high G protein-coupled receptor protein (hGR3) expression CHO cell line.

The resulting CHO cells are cultured in a nucleic

acid-free screening medium in a CO_2 incubator at 37℃ using 5% CO_2 for 1-4 days so as to give the G protein-coupled receptor protein (hGR3).

The G protein-coupled receptor protein is purified from the above CHO cells using an affinity column prepared by conjugating an antibody to the G protein-coupled receptor protein or a partial peptide thereof to a support or an affinity column prepared by conjugating a ligand for the G protein-coupled receptor protein.

The activity of the G protein-coupled receptor protein thus formed can be measured by experimenting the binding with a ligand or by enzyme immunoassays using specific antibodies.

Hereinafter, a method for determing a ligand to the G protein-coupled receptor protein is described in detail.

The G protein-coupled receptor protein, the partial peptide thereof or a salt thereof is useful as a reagent for investigating or determining a ligand to said G protein-coupled receptor protein.

According to the present invention, methods for determining a ligand to the G protein-coupled receptor protein which comprises contacting the G protein-coupled receptor protein or the partial peptide thereof with the compound to be tested, and measuring the binding amount, the cell stimulating activity, etc. of the test compound to the G protein-coupled receptor protein or the partial peptide thereof are provided.

The compound to be tested may include not only known ligands such as angiotensins, bombesins, canavinoids, cholecystokinins, glutamine, serotonin, melatonins, neuropeptides Y, opioids, purine, vasopressins, oxytocins, VIP (vasoactive intestinal and related peptides), somatostatins, dopamine, motilins, amylins, bradykinins, CGRP (calcitonin gene related

peptides), leukotrienes, pancreastatins, prostaglandins, thromboxanes, adenosine, adrenaline, α - and β - chemokines such as IL-8, GRO α, GRO β, GRO γ, NAP-2, ENA-78, PF4, IP10, GCP-2, MCP-1, HC14, MCP-3, I-309, MIP1 α, MIP-1 β, RANTES, etc.; endothelins, enterogastrins, histamine, neurotensins, TRH, pancreatic polypeptides, galanin, modified derivatives thereof, analogues thereof, family members thereof and the like but also tissue extracts, cell culture supernatants, etc. of human or warm-blooded aminals such as mice, rats, swines, cattle, sheep and monkeys, etc. For example, said tissue extract, said cell culture supernatant, etc. is added to the G protein-coupled receptor protein for measurement of the cell stimulating activity, etc. and fractionated by relying on the measurements whereupon a single ligand can be finally determined and obtained.

In one specific embodiment of the present invention, said method for determining the ligand includes a method for determining whether a sample (including a compound or a salt thereof) is capable of stimulating a target cell which comprises binding said compound with the G protein-coupled receptor protein either in the presence of the G protein-coupled receptor protein, the partial peptide thereof or a salt thereof, or in a receptor binding assay system in which the expression system for the recombinant receptor protein is constructed and used; and measuring the receptor-mediated cell stimulating activity, etc. Examples of said cell stimulating activities that can be measured include promoting or inhibiting biological responses, e.g. liberation of arachidonic acid, liberation of acetylcholine, liberation of endocellular Ca^{2+}, production of endocellular cAMP, production of endocellular cGMP, production of inositol phosphate, changes in the cell membrane potential, phosphorylation

of endocellular protein, activation of c-fos, decrease in pH, etc, and preferably liberation of arachidonic acid. Examples of said compound or a salt thereof capable of stimulating the cell via binding with the G protein-coupled receptor protein include peptides, proteins, nonpeptidic compounds, synthetic compounds, fermented products, etc.

In more specific embodiments of the present invention, said methods for screening and identifying a ligand includes:

1) a method of screening for a ligand to a G protein-coupled receptor protein, which comprises contacting a labeled test compound with a G protein-coupled receptor protein or a salt thereof or its partial peptide or a salt thereof, and measuring the amount of the labeled test compound binding with said protein or salt thereof or with said partial peptide or salt thereof;

2) a method of screening for a ligand to a G protein-coupled receptor protein, which comprises contacting a labeled test compound with cells containing the G protein-coupled receptor protein or the membrane fraction of said cell, and measuring the amount of the labeled test compound binding with said cells or said membrane fraction;

3) a method of screening for a ligand to a G protein-coupled receptor protein, which comprises contacting a labeled test compound with the G protein-coupled receptor protein expressed on cell membranes by culturing transformants carrying the G protein-coupled receptor protein-encoding DNA and measuring the amount of the labeled test compound binding with said G protein-coupled receptor protein;

4) a method of screening for a ligan to a G protein-coupled receptor protein, which comprises contacting a test compound with cells containing the G protein-coupled receptor protein, and measuring the cell

stimulating activity, e.g. promoting or inhibiting activity on biological responses such as liberation of arachidonic acid, liberation of acetylcholine, liberation of endocellular Ca^{2+}, production of endocullular cAMP, production of endocellular cGMP, production of inositol phosphate, changes in the cell membrane potential, phosphorylation of endocellular protein, activation of c-fos, lowering in pH, etc. via the G protein-coupled receptor protein; and

5) a method of screening for a ligand to the G protein-coupled receptor protein, which comprises contacting a test compound with the G protein-coupled receptor protein expressed on the cell membrane by culturing transformants carrying the G protein-coupled receptor protein-encoding DNA, and measuring at least one cell stimulating activity, e.g., an activity for promoting or inhibiting physiological responses such as liberation of arachidonic acid, liberation of acetylcholine, liberation of endocellular Ca^{2+}, production of endocellular cAMP, production of endocellular cGMP, production of inositol phosphate, changes in the cell membrane potential, phosphorylation of endocellular protein, activation of c-fos, lowering in pH etc. via the G protein-coupled receptor protein.

Described below are specific illustrations of the method for screening and identifying ligands.

First, the G protein-coupled receptor protein used for the method for determining the ligand may include any material so far as it contains a G protein-coupled receptor protein, a partial peptide thereof or a salt thereof although it is preferable to express large amounts of the G protein-coupled receptor proteins in animal cells.

In the manufacture of the G protein-coupled receptor protein, the above-mentioned method can be used and carried out by expressing said protein

encoding DNA in mammalian cells or in insect cells. With respect to the DNA fragment coding for a particular region such as an extracellular epitope, the extracellular domains, etc., complementary DNA may be used although the method of expression is not limited thereto. For example, gene fragments or synthetic DNA may be used as well.

In order to introduce the G protein-coupled receptor protein-encoding DNA fragment into host animal cells and to express it efficiently, it is preferred that said DNA fragment is incorporated into the downstream side of polyhedron promoters derived from nuclear polyhedrosis virus belonging to baculovirus, promoters derived from SV40, promoters derived from retrovirus, metallothionein promoters, human heat shock promoters, cytomegalovirus promoters, SR α promoters, etc. Examinations of the quantity and the quality of the expressed receptor can be carried out by methods per se known to those of skill in the art or methods similar thereto based upon the present disclosure. For example, they may be conducted by methods described in publications such as Nambi, P. et al: The Journal of Biochemical Society, vol.267, pages 19555-19559 (1992).

Accordingly, with respect to the determination of the ligand, the material containing a G protein-coupled receptor protein or partial peptide thereof may include products containing G protein-coupled receptor proteins which are purified by methods per se known to those of skill in the art or methods similar thereto, peptide fragments of said G protein-coupled receptor protein, cells containing said G protein-coupled receptor protein, membrane fractions of the cell containing said protein, etc.

When the G protein-coupled receptor protein-containing cell is used in the determining method of the ligand, said cell may be immobilized with binding

agents including glutaraldehyde, formalin, etc. The immobilization may be carried out by methods per se known to those of skill in the art or methods similar thereto.

The G protein-coupled receptor protein-containing cells are host cells which express the G protein-coupled receptor protein. Examples of said host cells are microorganisms such as Escherichia coli, Bacillus subtilis, yeasts, insect cells, animal cells, etc.

The cell membrane fraction is a cell membrane-rich fraction which is prepared by methods per se known to those of skill in the art or methods similar thereto after disruption of cells. Examples of cell disruption may include a method for squeezing cells using a Potter-Elvehjem homogenizer, a disruption by a Waring blender or a Polytron manufactured by Kinematica, a disruption by ultrasonic waves, a disruption via blowing out cells from small nozzles together with applying a pressure using a French press or the like, etc. In the fractionation of the cell membrane, a fractionation method by means of centrifugal force such as a fractional centrifugal separation and a density gradient centrifugal separation is mainly used. For example, disrupted cellular liquid is centrifuged at a low speed (500 rpm to 3,000 rom) for a short period (usually, from about one to ten minutes), the supernatant liquid is further centrifuged at a high speed (15,000 rpm to 30,000 rpm) usually for 30 minutes to two hours and the resulting precipitate is used as a membrane fraction. Said membrane fraction contains a lot of the expressed G protein-coupled receptor protein and a lot of membrane components such as phospholipids and membrane proteins derived from the cells.

The amount of the G protein-coupled receptor protein in the membrane fraction cell containing said G protein-coupled receptor protein is preferably 10^3 to

10^8 molecules per cell or, more preferably, 10^5 to 10^7 molecules per cell. Incidentally, the greater the expressed amount, the higher the ligand binding activity (specific activity) per membrane fraction whereby the construction of a highly sensitive screening system becomes possible and, moreover, it permits measurement of a large amount of samples within the same lot.

In conducting the above-mentioned methods 1) to 3) wherein ligands capable of binding with the G protein-coupled receptor protein are determined, a suitable G protein-coupled receptor fraction and a labeled test compound are necessary. The G protein-coupled receptor fraction is preferably a naturally occurring (natural type) G protein-coupled receptor, a recombinant G protein-coupled receptor having the activity equivalent to that of the natural type. Here, the term "activity equivalent to" means the equivalent ligand binding activity, etc. as discussed above.

Suitable examples of the labeled test compound include above-mentioned compound to be tested which are labeled with [^{3}H], [^{125}I], [^{14}C], [^{35}S], etc.

Specifically, the determination of ligands capable of binding with G protein-coupled receptor proteins is carried out as follows:

First, cells or cell membrane fractions containing the G protein-coupled receptor protein are suspended in a buffer suitable for the assay to prepare the receptor sample for conducting the method of determining the ligand binding with the G protein-coupled receptor protein. The buffer may include any buffer such as Tris-HCl buffer or phosphate buffer with pH 4-10, preferably, pH 6-8, etc., as long as it does not inhibit the binding of the ligand with the receptor. In addition, surface-active agents such as CHAPS, Tween 80™ (Kao-Atlas, Japan), digitonin, deoxycholate, etc.

and various proteins such as bovine serum albumin(BSA), gelatin, milk derivatives, etc. may be added to the buffer with an object of descreasing the non-specific binding. Further, a protease inhibitor such as PMSF, leupeptin, E-64 (manufactured by Peptide Laboratory), pepstatin, etc. may be added with an object of inhibiting the decomposition of the receptor and the ligand by protease. A test compound labeled with a predetermined (or certain) amount (5,000 cpm to 500,000 cpm) of [^{3}H], [^{125}I]. [^{14}C], [^{35}S], etc. coexists in 0.01 ml to 10 ml of said receptor solution. In order to know the non-specific binding amount (NSB), a reaction tube to which a great excessive amount of the unlabeled test compound is added is prepared as well. The reaction is carried out at 0-50℃, preferably at 4-37℃ for 20 minutes to 24 hours, preferably 30 minutes to three hours. After the reaction, it is filtered through a glass fiber filter or the like, washed with a suitable amount of the same buffer and the radioactivity remaining in the glass fiber filter is measured by means of a liquid scintillation counter or a gamma-counter. The test compound in which the count (B - NSB) obtained by subtracting the non-specific binding amount (NSB) from the total binding amount (B) is more than 0 cpm is identified as a ligand to the G protein-coupled receptor protein.

In conducting the above-mentioned methods 4) to 5) wherein ligands capable of binding with the G protein-coupled receptor protein are determined, the cell stimulating activity, e.g. the liberation of arachidonic acid, the liberation of acetylcholine, endocellular Ca^{2+} liberation, endocellular cAMP production, the production of inositol phosphate, changes in the cell membrane potential, the phosphorylation of endocellular protein, the activation of c-fos, lowering of pH, the activation of G protein,

cell promulgation, etc.; mediated by the G protein-coupled receptor protein may be measured by known methods or by the use of commercially available measuring kits. To be more specific, G protein-coupled receptor protein-containing cells are at first cultured in a multi-well plate or the like.

In conducting the determination of ligand, it is substituted with a fresh medium or a suitable buffer which does not show toxicity to the cells in advance of the experiment, and incubated under appropriate conditions and for sufficient time after adding a test compound, etc. thereto. Then, the cells are extracted or the supernatant liquid is recovered and the resulting product is determined by each of the methods. When it is difficult to identify the production of the substance, e.g. arachidonic acid, etc. which is to be an index for the cell stimulating activity due to the decomposing enzyme contained in the cell, an assay may be carried out by adding an inhibitor against said decomposing enzyme. With respect to an activity such as an inhibitory action against cAMP production, it may be detected as an inhibitory action against the production of the cells whose fundamental production is increased by forskolin or the like.

The kit used for the method of determining the ligand binding with the G protein-coupled receptor protein includes a G protein-coupled receptor protein or a partial peptide thereof, cells containing the G protein-coupled receptor protein, a membrane fraction from the cells containing the G protein-coupled receptor protein, etc.

Examples of the kit for determining the ligand are as follows:

1. Reagent for Determing the Ligand.

1) Buffer for Measurement and Buffer for Washing.

The buffering product wherein 0.05% of bovine serum

albumin (manufactured by Sigma) is added to Hanks' Balanced Salt Solution (manufactured by Gibco).

This product may be sterilized by filtration through a membrane filter with a 0.45 μm pore size, and stored at 4℃ or may be formulated upon use.

2) G protein-coupled receptor Protein Sample.

CHO cells in which G protein-coupled receptor proteins are expressed are subcultured at the rate of 5 x 10^5 cells/well in a 12-well plate and cultured at 37℃ in a humidified 5% CO_2/95% air atmosphere for two days to prepare the sample.

3) Labeled Test Compound.

The compound which is labeled with commercially available [^{3}H], [^{125}I], [^{14}C], [^{35}S], etc. or labeled with a suitable method.

The product in a state of an aqueous solution is stored at 4℃ or at -20℃ and, upon use, diluted to 1 μM with a buffer for the measurement. In the case of a test compound which is barely soluble in water, it may be dissolved in an organic solvent such as dimethylformamide, DMSO, methanol and the like.

4) Unlabeled Test Compound.

The same compound as the labeled one is prepared in a concentration of 100 to 1,000-fold concentrated state.

2. Method of Measurement

1) G protein-coupled receptor protein-expressing CHO cells cultured in a 12-well tissue culture plate are washed twice with 1 ml of buffer for the measurement and then 490 μl of buffer for the measurement is added to each well.

2) Five μl of the labeled test compound is added and the mixture is made to react at room temperature for one hour. For measuring the nonspecific binding amount, 5 μl of the unlabeled test compound is added.

3) The reaction solution is removed from each well, which is washed with 1 ml of a buffer for the

measurement three times. The labeled test compound which is binding with the cells is dissolved in 0.2N NaOH-1% SDS and mixed with 4 ml of a liquid scintillator A manufactured by WAKO Pure Chemical, Japan.

4) Radioactivity is measured using a liquid scintillation counter such as one manufactured by Beckmann.

Each SEQ ID NO set forth in the SEQUENCE LISTING of the specification refers to the following sequence:

[SEQ ID NO:1] is an entire amino acid sequence of the bovine pituitary-derived ligand polypeptide encoded by the cDNA included in pBOV3.

[SEQ ID NO:2] is an entire nucleotide sequence of the bovine pituitary-derived ligand polypeptide cDNA.

[SEQ ID NO:3] is an amino acid sequence of the bovine pituitary-derived ligand polypeptide which was obtained by purification and analysis of N-terminal sequence for P-3 fraction. The amino acid sequence corresponds to 23rd to 51st positions of the amino acid sequence of SEQ ID NO:1. [SEQ ID NO:4] is an amino acid sequence of the bovine pituitary-derived ligand polypeptide which was obtained by purification and analysis of N-terminal sequence for P-2 fraction. The amino acid sequence corresponds to 34th to 52nd positions of the amino acid sequencce of SEQ ID NO:1.

[SEQ ID NO:5] is an amino acid sequence of the bovine pituitary-derived ligand polypeptide. The amino acid sequence corresponds to 23rd to 53rd positions of the amino acid sequence of SEQ ID NO:1.

[SEQ ID NO:6] is an amino acid sequence of the bovine pituitary-derived ligand polypeptide. The amino acid sequence corresponds to 23rd to 54th positions of the amino acid sequence of SEQ ID NO:1.

[SEQ ID NO:7] is an amino acid sequence of the bovine pituitary-derived ligand polypeptide. The amino acid

sequence corresponds to 23rd to 55th positions of the amino acid sequence of SEQ ID NO:1.

[SEQ ID NO:8] is an amino acid sequence of the bovine pituitary-derived ligand polypeptide. The amino acid sequence corresponds to 34th to 53rd positions of the amino acid sequence of SEQ ID NO:1.

[SEQ ID NO:9] is an amino acid sequence of the bovine pituitary-derived ligand polypeptide. The amino acid sequence corresponds to 34th to 54th positions of the amino acid sequence of SEQ ID NO:1.

[SEQ ID NO:10] is an amino acid sequence of the bovine pituitary-derived ligand polypeptide. The amino acid sequence corresponds to 34th to 55th positions of the amino acid sequence of SEQ ID NO:1.

[SEQ ID NO:11] is a nucleotide sequence of DNA coding for the bovine pituitary-derived ligand polypeptide (SEQ ID NO:3).

[SEQ ID NO:12] is a nucleotide sequence of DNA coding for the bovine pituitary-derived ligand polypeptide (SEQ ID NO:4).

[SEQ ID NO:13] is a nucleotide sequence of DNA coding for the bovine pituitary-derived ligand polypeptide (SEQ ID NO:5).

[SEQ ID NO:14] is a nucleotide sequence of DNA coding for the bovine pituitary-derived ligand polypeptide (SEQ ID NO:6).

[SEQ ID NO:15] is a nucleotide sequence of DNA coding for the bovine pituitary-derived ligand polypeptide (SEQ ID NO:7).

[SEQ ID NO:16] is a nucleotide sequence of DNA coding for the bovine pituitary-derived ligand polypeptide (SEQ ID NO:8).

[SEQ ID NO:17] is a nucleotide sequence of DNA coding for the bovine pituitary derived ligand polypeptide (SEQ ID NO:9).

[SEQ ID NO:18] is a nucleotide sequence of DNA coding

for the bovine pituitary-derived ligand polypeptide (SEQ ID NO:10).

[SEQ ID NO:19] is a partial amino acid sequence of the human pituitary-derived G protein-coupled receptor protein encoded by the human pituitary-derived G protein-coupled receptor protein cDNA fragment included in p19P2.

[SEQ ID NO:20] is a partial amino acid sequence of the human pituitary-derived G protein-coupled receptor protein encoded by the human pituitary-derived G protein-coupled receptor protein cDNA fragment include in p19P2.

[SEQ ID NO:21] is an entire amino acid sequence of the human pituitary-derived G protein-coupled receptor protein encoded by the human pituitary-derived G protein-coupled receptor protein cDNA include in phGR3.

[SEQ ID NO:22] is a partial amino acid sequence of the mouse pancreatic β-cell line, MIN6-derived G protein-coupled receptor protein encoded by the mouse pancreatic β-cell line, MIN6-derived G protein-coupled receptor protein cDNA fragment having a nucleotide sequence (SEQ ID NO:27), derived based upon the nucleotide sequences of the mouse pancreatic β-cell line, MIN6-derived G protein-coupled receptor protein cDNA fragments each included in pG3-2 and pG1-10.

[SEQ ID NO:23] is a partial amino acid sequence of the mouse pancreatic β-cell line, MIN6-derived G protein-coupled receptor protein encoded by p5S38.

[SEQ ID NO:24] is a nucleotide sequence of the human pituitary-derived G protein-coupled receptor protein cDNA fragment include in p19P2.

[SEQ ID NO:25] is a nucleotide sequence of the human pituitary-derived G protein-coupled receptor protein cDNA fragment include in p19P2.

[SEQ ID NO:26] is an entire nucleotide sequence of the human pituitary-derived G protein-coupled receptor

protein cDNA include in phGR3.

[SEQ ID NO:27] is a nucleotide sequence of the mouse pancreatic β-cell line, MIN6-derived G protein-coupled receptor protein cDNA, derived based upon the nucleotide sequences of the mouse pancreatic β-cell line, MIN6-derived G protein-coupled receptor protein cDNA fragments each included in pG3-2 and pG1-10.

[SEQ ID NO: 28] is a nucleotide sequence of the mouse pancreatic β-cell line, MIN6-derived G protein-coupled receptor protein cDNA include in p5S38.

[SEQ ID NO:29] is a synthetic DNA primer for screening of cDNA coding for the G protein-coupled receptor protein.

[SEQ ID NO:30] is a synthetic DNA primer for screening of cDNA coding for the G protein-coupled receptor protein.

[SEQ ID NO:31] is a synthetic DNA primer for screening of cDNA coding for the G protein-coupled receptor protein.

[SEQ ID NO:32] is a synthetic DNA primer for screening of cDNA coding for the G protein-coupled receptor protein.

[SEQ ID NO:33] is a synthetic DNA primer for screening of cDNA coding for the G protein-coupled receptor protein.

[SEQ ID NO:34] is a synthetic DNA primer for screening of cDNA coding for the G protein-coupled receptor protein.

[SEQ ID NO:35] is a synthetic DNA primer for screening of cDNA coding for the bovine pituitary-derived ligand polypeptide, wherein the primer is represented by P5-1.

[SEQ ID NO:36] is a synthetic DNA primer for screening of cDNA coding for the bovine pituitary-derived ligand polypeptide, wherein the primer is represented by P3-1.

[SEQ ID NO:37] is a synthetic DNA primer for screening of cDNA coding for the bovine pituitary-derived ligand

polypeptide, wherein the primer is represented by P3-2.
[SEQ ID NO:38] is a synthetic DNA primer for screening of cDNA coding for the bovine pituitary-derived ligand polypeptide, wherein the primer is represented by PE.
[SEQ ID NO:39] is a synthetic DNA primer for screening of cDNA coding for the bovine pituitary-derived ligand polypeptide, wherein the primer is represented by PDN.
[SEQ ID NO:40] is a synthetic DNA primer for screening of cDNA coding for the bovine pituitary-derived ligand polypeptide, wherein the primer is represented by FB.
[SEQ ID NO:41] is a synthetic DNA primer for screening of cDNA coding for the bovine pituitary-derived ligand polypeptide, wherein the primer is represented by FC.
[SEQ ID NO:42] is a synthetic DNA primer for screening of cDNA coding for the bovine pituitary-derived ligand polypeptide, wherein the primer is represented by BOVF.
[SEQ ID NO:43] is a synthetic DNA primer for screening of cDNA coding for the bovine pituitary-derived ligand polypeptide, wherein the primer is represented by BOVR.
[SEQ ID NO:44] is an entire amino acid sequence of the bovine genome-derived ligand polypeptide.
[SEQ ID NO: 45] is an entire amino acid sequence of the rat type ligand polypeptide encoded by the cDNA included in pRAV3.
[SEQ ID NO:46] is an entire nucleotide sequence of the rat type ligand polypeptide cDNA.
[SEQ ID NO:47] is an amino acid sequence of the rat type ligand polypeptide. The amino acid sequence corresponds to 22nd to 52nd positions of the amino acid sequence of SEQ ID NO:45.
[SEQ ID NO:48] is an amino acid sequence of the rat type ligand polypeptide. The amino acid sequence corresponds to 22nd to 53rd positions of the amino acid sequence of SEQ ID NO:45.
[SEQ ID NO:49] is an amino acid sequence of the rat type ligand polypeptide. The amino acid sequence

corresponds to 22nd to 54th positions of the amino acid sequence of SEQ ID NO:45.

[SEQ ID NO:50] is an amino acid sequence of the rat type ligand polypeptide. The amino acid sequence corresponds to 33rd to 52nd positions of the amino acid sequence of SEQ ID NO:45.

[SEQ ID NO:51] is an amino acid sequence of the rat type ligand polypeptide. The amino acid sequence corresponds to 33rd to 53rd positions of the amino acid sequence of SEQ ID NO:45.

[SEQ ID NO:52] is an amino acid sequence of the rat type ligand polypeptide. The amino acid sequence corresponds to 33rd to 54th positions of the amino acid sequence of SEQ ID NO.45.

[SEQ ID NO:53] is a nucleotide sequence encoding for the rat type ligand polypeptide of SEQ ID NO:47.

[SEQ ID NO:54] is a nucleotide sequence encoding for the rat type ligand polypeptide of SEQ ID NO:48.

[SEQ ID NO:55] is a nucleotide sequence encoding for the rat type ligand polypeptide of SEQ ID NO:49.

[SEQ ID NO:56] is a nucleotide sequence encoding for the rat type ligand polypeptide of SEQ ID NO:50.

[SEQ ID NO:57] is a nucleotide sequence encoding for the rat type ligand polypeptide of SEQ ID NO:51.

[SEQ ID NO:58] is a nucleotide sequence encoding for the rat type ligand polypeptide of SEQ ID NO:52.

[SEQ ID NO:59] is an entire amino acid sequence of the human type ligand polypeptide encoded by the cDNA includedin pHOB7.

[SEQ ID NO:60] is an entire nucleotide sequence of the human type ligand polypeptide cDNA.

[SEQ ID NO:61] is an amino acid sequence of the human type ligand polypeptide. The amino acid sequence corresponds to 23rd to 53rd positions of the amino acid sequence of SEQ ID NO.59.

[SEQ ID NO:62] is an amino acid sequence of the human

type ligand polypeptide. The amino acid sequence corresponds to 23rd to 54th positions of the amino acid sequence of SEQ ID NO.59.

[SEQ ID NO:63] is an amino acid sequence of the human type ligand polypeptide. The amino acid sequence corresponds to 23rd to 55th positions of the amino acid sequence of SEQ ID NO.59.

[SEQ ID NO:64] is an amino acid sequence of the human type ligand polypeptide. The amino acid sequence corresponds to 34th to 53rd positions of the amino acid sequence of SEQ ID NO.59.

[SEQ ID NO:65] is an amino acid sequence of the human type ligand polypeptide. The amino acid sequence corresponds to 34th to 54th positions of the amino acid sequence of SEQ ID NO.59.

[SEQ ID NO:66] is an amino acid sequence of the human type ligand polypeptide. The amino acid sequence corresponds to 34th to 55th positions of the amino acid sequence of SEQ ID NO.59.

[SEQ ID NO:67] is a nucleotide sequence encoding for the human type ligand polypeptide of SEQ ID NO:61.

[SEQ ID NO:68] is a nucleotide sequence encoding for the human type ligand polypeptide of SEQ ID NO:62.

[SEQ ID NO:69] is a nucleotide sequence encoding for the human type ligand polypeptide of SEQ ID NO:63.

[SEQ ID NO:70] is a nucleotide sequence encoding for the human type ligand polypeptide of SEQ ID NO:64.

[SEQ ID NO:71] is a nucleotide sequence encoding for the human type ligand polypeptide of SEQ ID NO:65.

[SEQ ID NO:72] is a nucleotide sequence encoding for the human type ligand polypeptide of SEQ ID NO:66.

[SEQ ID NO:73] is a partial amino acid sequence of the ligand polypeptide, wherein Xaa of the 10th position is Ala or Thr, Xaa of the 11th position is Gly or Ser and Xaa of the 21st position is H, Gly or GlyArg.

[SEQ ID NO:74] is a partial amino acid sequence of the

ligand polypeptide, wherein Xaa of the 3rd position is Ala or Thr, Xaa of the 5th position is Gln or Arg and Xaa of the 10th position is Ile or Thr.

[SEQ ID NO:75] is a synthetic DNA primer for screening of cDNA coding for the rat type ligand polypeptide, wherein the primer is represented by RA.

[SEQ ID NO:76] is a synthetic DNA primer for screening of cDNA coding for the rat type ligand polypeptide, wherein the primer is represented by RC.

[SEQ ID NO:77] is a synthetic DNA primer for screening of cDNA coding for the rat type ligand polypeptide, wherein the primer is represented by rF.

[SEQ ID NO:78] is a synthetic DNA primer for screening of cDNA coding for the rat type ligand polypeptide, wherein the primer is represented by rR.

[SEQ ID NO:79] is a synthetic DNA primer for screening of cDNA coding for the human type ligand polypeptide, wherein the primer is represented by R1.

[SEQ ID NO:80] is a synthetic DNA primer for screening of cDNA coding for the human type ligand polypeptide, wherein the primer is represented by R3.

[SEQ ID NO:81] is a synthetic DNA primer for screening of cDNA coding for the human type ligand polypeptide, wherein the primer is represented by R4.

[SEQ ID NO:82] is a synthetic DNA primer for screening of cDNA coding for the human type ligand polypeptide, wherein the primer is represented by HA.

[SEQ ID NO:83] is a synthetic DNA primer for screening of cDNA coding for the human type ligand polypeptide, wherein the primer is represented by HB.

[SEQ ID NO:84] is a synthetic DNA primer for screening of cDNA coding for the human type ligand polypeptide, wherein the primer is represented by HE.

[SEQ ID NO:85] is a synthetic DNA primer for screening of cDNA coding for the human type ligand polypeptide, wherein the primer is represented by HF.

[SEQ ID NO:86] is a synthetic DNA primer for screening of cDNA coding for the human type ligand polypeptide, wherein the primer is represented by 5H.

[SEQ ID NO:87] is a synthetic DNA primer for screening of cDNA coding for the human type ligand polypeptide, wherein the primer is represented by 3HN.

[SEQ ID NO:88] is a synthetic DNA primer for screening of cDNA coding for the rat type G protein-coupled receptor protein (UHR-1), wherein the primer is represented by rRECF.

[SEQ ID NO:89] is a synthetic DNA primer for screening of cDNA coding for the rat type G protein-coupled receptor protein (UHR-1), wherein the primer is represented by rRECR.

[SEQ ID NO:90] is a synthetic DNA which is used for amplification of G3PDH, UHR-1 and ligand, wherein the primer represented by r19F.

[SEQ ID NO:91] is a synthetic DNA which is used for amplification of G3PDH, UHR-1 and ligand, wherein the primer represented by r19R.

[SEQ ID NO:92] is a N-terminal peptide of the ligand polypeptide, which is used for antigen. (Peptide-I)

[SEQ ID NO:93] is a C-terminal peptide of the ligand polypeptide, which is used for antigen. (Peptide-II)

[SEQ ID NO:94] is a peptide of the central portion in ligand polypeptide, which is used for antigen. (Peptide-III)

[SEQ ID NO:95] is a synthetic DNA primer for screening of cDNA coding for rat type G protein-coupled receptor protein (UHR-1).

[SEQ ID NO:96] is a synthetic DNA primer for screening of cDNA coding for rat type G protein-coupled receptor protein (UHR-1).

[SEQ ID NO:97] is a synthetic DNA primer used in Example 48.

[SEQ ID NO:98] is a synthetic DNA primer used in

Example 48.

[SEQ ID NO:99] is a synthetic DNA prove used in Example 48.

The transformant Escherichia coli, designated INVα F'/p19P2, which is obtained in the Example 2 mentioned herein below, is on deposit under the terms of the Budapest Treaty from August 9, 1994, with the National Institute of Bioscience and Human-Technology (NIBH), Agency of Industrial Science and Technology, Ministry of International Trade and Industry, Japan and has been assigned the Accession Number FERM BP-4776. It is also on deposit from August 22, 1994 with the Institute for Fermentation, Osaka, Japan (IFO) and has been assigned the Accession Number IFO 15739.

The transformant Escherichia coli, designated INVα F'/pG3-2, which is obtained in the Example 4 mentioned herein below, is on deposit under the terms of the Budapest Treaty from August 9, 1994, with NIBH and has been assigned the Accession Number FERM BP- 4775. It is also on deposit from August 22, 1994 with IFO and has been assigned the Accession Number IFO 15740.

The transformant Escherichia coli, designated JM109/phGR3, which is obtained in the Example 5 mentioned herein below, is on deposit under the terms of the Budapest Treaty from September 27, 1994, with NIBH and has been assigned the Accession Number FERM BP-4807. It is also on deposit from September 22, 1994 with IFO and has been assigned the Accession Number IFO 15748.

The transformant Escherichia coli, designated JM109/p5S38, which is obtained in the Example 8 mentioned herein below, is on deposit under the terms of the Budapest Treaty from October 27, 1994, with NIBH and has been assigned the Accession Number FERM BP-4856. It is also on deposit from October 25, 1994 with

IFO and has been assigned the Accession Number IFO 15754.

The transformant Escherichia coli, designated JM109/pBOV3, which is obtained in the Example 20 mentioned herein below, is on deposit under the terms of the Budapest Treaty from February 13, 1996, with NIBH and has been assigned the Accession Number FERM BP-5391. It is also on deposit from January 25, 1996 with IFO and has been assigned the Accession Number IFO 15910.

The transformant Escherichia coli, designated JM109/pRAV3, which is obtained in the Example 29 mentioned herein below, is on deposit under the terms of the Budapest Treaty from September 12, 1996, with NIBH and has been assigned the Accession Number FERM BP-5665. It is also on deposit from September 3, 1996 with IFO and has been assigned the Accession Number IFO 16012.

The transformant Escherichia coli, designated JM109/pHOV7, which is obtained in the Example 32 mentioned herein below, is on deposit under the terms of the Budapest Treaty from September 12, 1996, with NIBH and has been assigned the Accession Number FERM BP-5666. It is also on deposit from September 5, 1996 with IFO and has been assigned the Accession Number IFO 16013.

[Industrial Application]

The ligand polypeptide of the present invention has prolactin secretion modulating activity, i.e. prolactin secretion promoting and/or inhibiting activities. Thus, as will be understood from the Examples presented hereinafter, the ligand polypeptide of the present invention has prolactin secretion promoting activity and, therefore, finds application as a drug for preventing and/or treating various diseases associated

with prolactin hyposecretion. On the other hand, the ligand polypeptide of the invention has a high affinity for the receptor proteins and, therefore, when used in an increased dose, causes desensitization for prolactin secretion, thus exhibiting prolactin secretion inhibiting activity. In this sense, it can be used as a drug for preventing and/or treating various diseases associated with prolactin hypersecretion.

Therefore, the ligand polypeptide of the invention can be used with advantage as a prolactin secretion-stimulating agent for the prevention and treatment of certain diseases associated with prolactin secretion, such as hypoovarianism, gonecyst cacogenesis, menopausal symdrome, euthyroid hypometabolism. In addition, the ligand polypeptide of the invention can be used with advantage as a aphrodisiac.

On the other hand, the ligand polypeptide of the invention can be used with advantage as a prolactin secretion inhibitory agent in the prevention and treatment of certain diseases associated with prolactin secretion, such as pituitary adenomatosis, brain tumor, emmeniopathy, autoimmune disease, prolactinoma, infertility, impotence, amenorrhea, galactorrhea, acromegaly, Chiari-Frommel symdrome, Argonz-del Castilo symdrome, Forbes-Albright symdrome, lymphoma, Sheehan syndrome or dyszoospermia.

In addition, the ligand poltpeptide of the present invention is used as a contraceptives based on its prolactin secretion inhibitory activity.

In addition, the ligand polypeptide of the invention can be used as a test reagent for study of the prolactin secretory function or a veterinary drug for use as a lactogogue in mammalian farm animals such as bovine, goat, and swine, and is even expected to find application in the elaboration of useful substances in such farm mammals and harvesting of the

substances secreted into their milk.

In addition, the ligand polypeptide of the present invention has a function of modulating placental function, and can be used as an agent for treating or preventing chriocarcinomia, hydatid mole, irruption mole, abortion, unthrifty fetus, abnormal saccharometabolism, abnormal lipidmetabolism or oxytocia.

[Examples]

Described below are working examples of the present invention which are provided only for illustrative purposes, and not to limit the scope of the present invention. [Reference Example 1]

Preparation of Synthetic DNA Primer for Amplifying DNA Coding for G protein-coupled receptor Protein

A comparitons of deoxyribonucleotide sequences coding for the known amino acid sequences corresponding to or near the first membrane-spanning domain each of human-derived TRH receptor protein (HTRHR), human-derived RANTES receptor protein (L10918, HUMRANTES), human Burkitt's lymphoma-derived unknown ligand receptor protein (X68149, HSBLR1A), human-derived somatostatin receptor protein (L14856, HUMSOMAT), rat-derived μ-opioid receptor protein (U02083, RNU02083), rat-derived κ-opioid receptor protein (U00442, U00442), human-derived neuromedin B receptor protein (M73482, HUMNMBR), human-derived muscarinic acetylcholine receptor protein (X15266, HSHM4), rat-derived adrenaline α_1B receptor protein (L08609, RATAADRE01), human-derived somatostatin 3 receptor protein (M96738, HUMSSTR3X), human-derived C_5a receptor protein (HUMC5AAR), human-derived unknown ligand receptor protein (HUMRDC1A), human-derived unknown ligand receptor protein (M84605, HUMOPIODRE) and rat-derived adrenaline α_2B receptor protein (M91466, RATA2BAR) was

made. As a result, highly homologous regions or parts were found.

Further, a comparison of deoxynucleotide sequences coding for the known amino acid sequences corresponding to or near the sixth membrane-spanning domain each of mouse-derived unknown ligand receptor protein (M80481, MUSGIR), human-derived bombesin receptor protein (L08893, HUMBOMB3S), human-derived adenosine A2 receptor protein (S46950, S46950), mouse-derived unknown ligand receptor protein (D21061, MUSGPCR), mouse-derived TRH receptor protein (S43387, S43387), rat-derived neuromedin K receptor protein (J05189, RATNEURA), rat-derived adenosine A1 receptor protein (M69045, RATA1ARA), human-derived neurokinin A receptor protein (M57414, HUMNEKAR), rat-derived adenosine A3 receptor protein (M94152, DATADENREC), human-derived somatostatin 1 receptor protein (M81829, HUMSRI1A), human-derived neurokinin 3 receptor protein (S86390, S86371S4), rat-derived unknown ligand receptor protein (X61496, RNCGPCR), human-derived somatostatin 4 receptor protein (L07061, HUMSSTR4Z) and rat-derived GnRH receptor protein (M31670, RATGNRHA) was made. As a result, highly homologous regions or parts were found.

The aforementioned abbreviations in the parentheses are identifiers (reference numbers) which are indicated when GenBank/EMBL Data Bank is retrieved by using DNASIS Gene/Protein Sequencing Data Base (CD019, Hitachi Software Engineering, Japan) and are usually called "Accession Numbers" or "Entry Names". HTRHR is, however, the sequence as disclosed in Japanese Patent Publication No. 304797/1993 (EPA 638645).

Specifically, it was planned to incorporate mixed bases relying upon the base regions that were in agreement with cDNAs coding for a large number of receptor proteins in order to enhance base agreement of sequences with as many receptor cDNAs as possible even

in other regions. Based upon these sequences, the degenerate synthetic DNA having a nucleotide sequence represented by SEQ ID NO:29 or SEQ ID NO:30 which is complementary to the homologous nucleotide sequence were produced. [Synthetic DNAs] 5'-CGTGG (G or C) C (A or C) T (G or C) (G or C) TGGGCAAC (A, G, C or T) (C or T) CCTG-3' (SEQ ID NO:29) 5'-GT (A, G, C or T) G (A or T) (A or G) (A or G) GGCA (A, G, C or T) CCAGCAGA (G or T) GGCAAA-3' (SEQ ID NO:30)

The parentheses indicate the incorporation of a plurality of bases, leading to multiple oligonucleotides in the primer preparation. In other words, nucleotide resides in parentheses of the aforementioned DNAs were incorporated in the presence of a mixture of plural bases at the time of synthesis.

[Example 1]

Amplification of Receptor cDNA by PCR Using Human Pituitary Gland-Derived cDNA

By using human pituitary gland-derived cDNA (QuickClone, CLONTECH Laboratories, Inc.) as a template, PCR amplification using the DNA primers synthesized in Reference Example 1 was carried out. The composition of the reaction solution consisted of the synthetic DNA primers (SEQ: 5' primer sequence and 3' primer sequence) each in an amount of 1 μM, 1ng of the template cDNA, 0.25 mM dNTPs, 1 μl of Taq DNA polymerase and a buffer attached to the enzyme kit, and the total amount of the reaction solution was made to be 100 μl. The cycle for amplification including 95℃ for 1 min., 55℃ for 1 min. and 72℃ for 1 min. was repeated 30 times by using a Thermal Cycler (Perkin-Elmer Co.). Prior to adding Taq DNA polymerase, the remaining reaction solution was mixed and was heated at 95℃ for 5 minutes and at 65℃ for 5 minutes. The amplified products were confirmed relying upon 1.2% agarose gel electrophoresis and ethidium bromide

staining.

[Example 2]

Subcloning of PCR Product into Plasmid Vector and Selection of Novel Receptor Candidate Clone via Decoding Nucleotide Sequence of Inserted cDNA Region

The PCR products were separated by using a 0.8% low-melting temperature agarose gel, the band parts were excised from the gel with a razor blade, and were heat-melted, extracted with phenol and precipitated in ethanol to recover DNAs. According to the protocol attached to a TA Cloning Kit (Invitrogen Co.), the recovered DNAs were subcloned into the plasmid vector, pCR™II (TM represents registered trademark). The recombinant vectors were introduced into E. coli INVα F' competent cells (Invitrogen Co.) to produce transformants. Then, transformant clones having a cDNA-inserted fragment were selected in an LB agar culture medium containing ampicillin and X-gal. Only transformant clones exhibiting white color were picked with a sterilized toothpick to obtain transformant Escherichia coli INVαF'/p19P2.

The individual clones were cultured overnight in an LB culture medium containing ampicillin and treated with an automatic plasmid extracting machine (Kurabo Co., Japan) to prepare plasmid DNAs. An aliquot of the DNA thus prepared was cut by EcoRI to confirm the size of the cDNA fragment that was inserted. An aliquot of the remaining DNA was further processed with RNase, extracted with phenol/chloroform, and precipitated in ethanol so as to be condensed. Sequencing was carried out by using a DyeDeoxy terminator cycle sequencing kit (ABI Co.), the DNAs were decoded by using a fluorescent automatic sequencer, and the data of the nucleotide sequences obtained were read by using DNASIS (Hitachi System Engineering Co., Japan). The underlined portions represent regions corresponding to the

synthetic primers.

Homology retrieval was carried out based upon the determined nucleotide sequences [SEQ ID NO:24 and 25 (Here, the determined nucleotide sequence is the nucleotide sequence which the underlined portion is deleted from the sequence of Figure 1 or Figure 2 respectively)].

As a result, it was learned that a novel G protein-coupled receptor protein was encoded by the cDNA fragment insert in the plasmid, p19P2, possessed by the transformant Escherichia coli INV α F'/p19P2. To further confirm this fact, by using DNASIS (Hitachi System Engineering Co., Japan) the nucleotide sequences were converted into amino acid sequences [SEQ ID NO:19 and 20], and homology retrieval was carried out in view of hydrophobicity plotting [Figures 3 and 4] and at the amino acid sequence level to find homology relative to neuropeptide Y receptor proteins [Figure 5].

[Example 3]

Preparation of Poly(A)$^+$RNA Fraction from Mouse Pancreatic β-Cell Strain, MIN6 and Synthesis of cDNA

A total RNA was prepared from the mouse pancreatic β-cell strain, MIN6 (Jun-ichi Miyazaki et al., Endocrinology, Vol. 127, No. 1, p.126-132) according to the guanidine thiocyanate method (Kaplan B.B. et al., Biochem. J., 183, 181-184 (1979) and, then, poly(A)$^+$RNA fractions were prepared with a mRNA purifying kit (Pharmacia Co.). Next, to 5 μg of the poly(A)$^+$RNA fraction was added a random DNA hexamer (BRL Co.) as a primer, and the resulting mixture was subjected to reaction with mouse Moloney Leukemia virus (MMLV) reverse transcriptase (BRL Co.) in the buffer attached to the MMLV reverse transcriptase kit to synthesize complementary DNAs. The reaction product was extracted with phenol/chloroform (1:1), precipitated in ethanol, and was then dissolved in 30 μl of TE buffer (10 mM

Tris-HCl at pH8.0, 1 mM EDTA at pH8.0).

[Example 4]

Amplification of Receptor cDNA by PCR Using MIN6-Derived cDNA and Sequencing

By suing, as a template, 5 μl of cDNA prepared from the mouse pancreatic β-cell strain, MIN6 in the above Example 3, PCR amplification using the DNA primers synthesized in Reference Example 1 was carried out under the same condition as in Example 1. The resulting PCR product was subcloned into the plasmid vector, pCRTMII, in the same manner as in Example 2 to obtain a plasmid, pG3-2. The plasmid pG3-2 was transfected into E. coli INVαF' to obtain transformed Escherichia coli INVαF'/pG3-2.

By using, as a template, 5 μl of the cDNA parepared from the mouse pancreatic β-cell strain, MIN6, PCR amplification using DNA primers as disclosed in Libert F. et al., "Science, 244:569-572, 1989", i.e., a degenerate synthetic primer represented by the following sequence: 5'-CTGTG (C or T) G (C or T) (G or C) AT (C or T) GCIIT (G or T) GA (C or T) (A or C) G (G or C) TAC-3'

(SEQ ID NO:31)

wherein I is inosine; and a degenerate synthetic primer represented by the following sequence: 5'-A (G or T) G (A or T) AG (A or T) AGGGCAGCCAGCAGAI (G or C) (A or G) (C or T) GAA-3' (SEQ ID NO:32) wherein I is inosine, was carried out under the same conditions as in Example 1. The resulting PCR product was subcloned into the plasmid vector, pCR™II, in the same manner as described in Example 2 to obtain a plasmid, pG1-10.

The reaction for determining the nucleotide sequence (sequencing) was carried out with a DyeDeoxy terminator cycle sequencing kit (ABI Co.), the DNA was decoded with the fluorescent automatic sequencer (ABI

Co.), and the data of the nucleotide sequence obtained were analyzed with DNASIS (Hitachi System Engineering Co., Japan).

Figure 6 shows a mouse pancreatic β-cell strain MIN6-derived G protein-coupled receptor protein-encoding DNA (SEQ ID NO:27) and an amino acid sequence (SEQ ID NO:22) encoded by the isolated DNA based upon the nucleotide sequences of plasmids pG3-2 and pG1-10 which are possessed by the transformant Escherichia coli INVαF'/pG3-2. The underlined portions represent regions corresponding to the synthetic primers.

Homology retrieval was carried out based upon the determined necleotide sequence [Figure 6]. As a result, it was learned that a novel G protein-coupled receptor protein was encoded by the cDNA fragment obtained. To further confirm this fact, by using DNASIS (Hitachi System Engineering Co., Japan) the nucleotide sequence was converted into an amino acid sequence [Figure 6], hydrophobicity plotting was carried out to confirm the presence of six hydrophobic regions [Figure 8]. Upon comparing the amino acid sequence with that of p19P2 obtained in Example 2, furthermore, a high degree of homology was found as shown in [Figure 7]. As a result, it is strongly suggested that the G protein-coupled receptor proteins encoded by pG3-2 and pG1-10 recognize the same ligand as the G protein-coupled receptor protein encoded by p19P2 while the animal species from which the receptor proteins encoded by pG3-2 and pG1-10 are derived is different from that from which the receptor protein encoded by p19P2 is.

[Example 5]

Cloning of cDNA Comprising Whole Coding Regions for Receptor Protein from Human Pituitary Gland-Derived cDNA Library

The DNA library constructed by Clontech Co. wherein λ gt11 phage vector is used (CLONTECH Laboratories,

Inc.; CLH L1139b) was employed as a human pituitary gland-derived cDNA library. The human pituitary gland cDNA library (2 x 10^6 pfu (plaque forming units)) was mixed with E. coli Y1090- treated with magnesium sulfate, and incubated at 37℃ for 15 minutes followed by addition of 0.5% agarose (Pharmacia Co.) LB. The E. coli was plated onto a 1.5% agar (Wako-Junyaku Co.) LB plate (containing 50 μg/ml of ampicillin). A nitrocellulose filter was placed on the plate on which plaques were formed and the plaque was transferred onto the filter. The filter was denatured with an alkali and then heated at 80℃ for 3 hours to fix DNAs.

The filter was incubated overnight at 42℃ together with the probe mentioned herein below in a buffer containing 50% formamide, 5 x SSPE (20 x SSPE (pH 7.4) is 3 M NaCl, 0.2 M $NaH_2PO_4.H_2O$, 25 mM EDTA), 5 X Denhardt's solution (Nippon Gene, Japan), 0.1% SDS and 100 μg/ml of salmon sperm DNA for hybridization.

The probe used was obtained by cutting the DNA fragment inserted in the plasmid, p19P2, obtained in Example 2, with EcoRI, followed by recovery and labelling by incorporation of [^{32}P]dCTP (Dupont Co.) with a random prime DNA labelling kit (Amasham Co.).

It was washed with 2 x SSC (20 x SSC is 3 M NaCl, 0.3 M sodium citrate), 0.1% SDS at 55℃ for 1 hour and, then, subjected to an autoradiography at -80 ℃ to detect hybridized plaques.

In this screening, hybridization signals were recognized in three independent plaques. Each DNA was prepared from the three clones. The DNAs digested with EcoRI were subjected to an agarose electrophoresis and were analyzed by the southern blotting using the same probe as the one used in the screening. Hybridizing bands were identified at about 0.7kb, 0.8kb and 2.0kb, respectively. Among them, the DNA fragment corresponding to the band at about 2.0kb (λ hGR3) was

selected. The λ hGR3-derived EcoRI fragment with a hybridizable size was subcloned to the EcoRI site of the plasmid, pUC18, and E. coli JM109 was transformed with the plasmid to obtain transformant E. coli JM109/phGR3. A restriction enzyme map of the plasmid, phGR3, was prepared relying upon a restriction enzyme map deduced from the nucleotide sequence as shown in Example 2. As a result, it was learned that it carried a full-length receptor protein-encoding DNA which was predicted from the receptor protein-encoding DNA as shown in Example 2.

[Example 6]

Sequencing of Human Pituitary Gland-Derived Receptor Protein cDNA

Among the EcoRI fragments inserted in the plasmid, phGR3, obtained in the above Example 5, the from EcoRI to NheI nucleotide sequence with about 1330bp that is considered to be a receptor protein-coding region was sequenced. Concretely speaking, by utilizing restriction enzyme sites that exist in the EcoRI fragments, unnecessary parts were removed or necessary fragments were subcloned in order to prepare template plasmids for analyzing the nucleotide sequence.

The reaction for determining the nucleotide sequence (sequencing) was carried out with a DyeDeoxy terminator cycle sequencing kit (ABI Co.), the DNA was decoded with the fluorescent automatic sequencer (ABI Co.), and the data of the nucleotide sequence obtained were analyzed with DNASIS (Hitachi System Engineering Co., Japan).

Figure 9 shows a nucleotide sequence of from immediate after the EcoRI site up to the NheI site encoded by phGR3. The nucleotide sequence of the human pituitary gland-derived receptor protein-encoding DNA corresponds to the nucleotide sequence (SEQ ID NO:26) of from 118th to 1227th nucleotides [Figure 9]. An

amino acid sequence of the receptor protein that is encoded by the nucleotide sequence is shown in SEQ ID NO:21.

[Example 7]

Northern Hybridization with Human Pituitary Gland-Derived Receptor Protein-Encoding phGR3

Northern blotting was carried out in order to detect the expression of phGR3-encoded human pituitary gland-derived receptor proteins obtained in Example 5 in the pituitary gland at a mRNA level. Human pituitary gland mRNA (2.5 μg, Clontech Co.) was used as a template mRNA and the same as the probe used in Example 5 was used as a probe. Nylon membrane (Pall Biodyne, U.S.A.) was used as a filter for northern blotting and migration of the mRNA and adsorption (sucking) thereof with the blotting filter was carried out according to the method as disclosed in Molecular Cloning, Cold Spring Harbor Laboratory Press, 1989.

The hybridization was effected by incubating the above-mentioned filter and probe in a buffer containing 50% formamide, 5 x SSPE, 5 X Denhardt's solution, 0.1% SDS and 100 μg/ml of salmon sperm DNA overnight at 42℃. The filter was washed with 0.1 x SSC, 0.1% SDS at 50℃ and, after drying with air, was exposed to an X-ray film (XAR5, Kodak) for three days at -80℃. The results were as shown in Figure 10 from which it is considered that the receptor gene encoded by phGR3 is expressed in the human pituitary gland.

[Example 8]

Amplification of Receptor cDNA by PCR Using MIN6-Derived cDNA and Sequencing

By using, as a template, 5 μl of cDNA prepared from the mouse pancreatic β-cell strain, MIN6 in Example 3, PCR amplification using the DNA primers synthesized in Example 4 as disclosed in Libert F. et al., "Science, 244:569-572, 1989", i.e., a synthetic

primer represented by the following sequence: 5'-CTGTG (C or T) G (C or T) (G or C) AT (C or T) GCIIT (G or T) GA (C or T) (A or C) G (G or C) TAC-3' (SEQ ID NO:31) wherein I is inosine; and a synthetic primer represented by the following sequence: 5'-A (G or T) G (A or T) AG (A or T) AGGGCAGCCAGCAGAI (G or C) (A or G) (C or T) GAA-3' (SEQ ID NO:32) wherein I is inosine, was carried out under the same conditions as in Example 1. The resulting PCR product was subcloned to the plasmid vector, pCRTMII, in the same manner as in Example 2 to obtain a plasmid, p5S38. The plasmid p5S38 was transfected into E. coli JM109 to obtain transformant Escherichia coli JM109/p5S38.

The reaction for determining the nucleotide sequence (sequencing) was carried out with a DyeDeoxy terminator cycle sequencing kit (ABI Co.), the DNA was decoded with the fluorescent automatic sequencer (ABI Co.), and the data of the nucleotide sequence obtained were read with DNASIS (Hitachi System Engineering Co., Japan).

Figure 12 showns a mouse pancreatic β-cell strain MIN6-derived G protein-coupled receptor protein-encoding DNA (SEQ ID NO:28) and an amino acid sequence (SEW ID NO:23) encoded by the isolated DNA based upon the nucleotide sequence of plasmid, p5S38. The underlined portions represent regions corresponding to the synthetic primers.

Homology retrieval was carried out based upon the determined nucleotide sequence [Figure 12]. As a result, it was learned that a novel G protein-coupled receptor protein was encoded by the cDNA fragment obtained. To further confirm this fact, by using DNASIS (Hitachi System Engineering Co., Japan), the nucleotide sequence was converted into an amino acid sequence [Figure 12], and hydrophobicity plotting was carried out to confirm the presence of four hydrophobic

regions [Figure 14]. Upon comparing the amino acid sequence with those encoded by p19P2 obtained in Example 2 and encoded by pG3-2 obtained in Example 4, furthermore, a high degree of homology was found as shown in Figure 13. As a result, it is strongly suggested that the mouse pancreatic β -cell strain, MIN6-derived G protein-coupled receptor protein encoded by p5S38 recognizes the same ligand as the human pituitary gland-derived G protein-coupled receptor protein encoded by p19P2 does while the animal species from which the receptor protein encoded by p5S38 is derived is different from that from which the receptor protein encoded by p19P2 is. It is also strongly suggested that the mouse pancreatic β -cell strain, MIN6-derived G protein-coupled receptor protein encoded by p5S38 recognized the same ligand as the mouse pancreatic β -cell strain, MIN6-derived G protein-coupled receptor proteins encoded by pG3-2 and pG1-10 do and they are analogous receptor proteins one another (so-called "subtype").

[Example 9]

Preparation of CHO cells which express phGR3

The plasmid phGR3 (Example 5) containing a cDNA encoding the full-length amino acid sequence of human pituitary receptor protein was digested with the restriction enzyme Nco I and electrophoresed on agarose gel and a fragment of about 1kb was recovered. Both ends of the recovered fragment were blunted with a DNA blunting kit (Takara Shuzo Co., Japan) and, with the SalI linker added, treated with SalI and inserted into the SalI site of pUC119 to provide plasmid S10. Then, S10 was treated with SalI and SacII to prepare a fragment of about 700 bp (containing the N-terminal coding region). Then, a fragment of about 700 bp (containing the C-terminal coding region including initiation and termination codons) was cut out from

phGR3 with Sac II and Nhe I. These two fragments were added to the animal cell expression vector plasmid pAKKO-111H (the vector plasmid identical to the pAKKO1.11 H described in Biochim. Biophys. Acta, Hinuma, S., et al., 1219 251-259, 1994) and a ligation reaction was carried out to construct a full-length receptor protein expression plasmid pAKKO-19P2.

E. coli transfected with pAKKO-19P2 was cultured and the pAKKO-19P2 plasmid DNA was mass-produced using QUIAGEN Maxi. A 20 μg portion of the plasmid DNA was dissolved in 1 ml of sterile PBS, and in a gene transfer vial (Wako Pure Chemical Ind.), the solution was vortexed well for liposome formation. This liposome, 125 μl, was added to CHOdhfr$^-$ cells subcultured at 1 x 10^6 per 10cm-dia. dish 24 hr before and placed in fresh medium immediately before addition and overnight culture was carried out. After a further one-day culture in fresh medium, the medium was changed to a screening medium and the incubation was further carried out for a day. For efficient screening of transformants, subculture was carried out at a low cell density and only the cells growing in the screening medium were selected to establish a full-length receptor protein expression CHO cell line CHO-19P2.

[Example 10]

Confirmation of the amount of expression of the full-length receptor protein in the CHO-19P2 cell line at the transcription level

Using FastTrack Kit (Invitrogen), CHO cells transfected with pAKKO-19P2 according to the kit manual and mock CHO cells were used to prepare poly(A)$^+$RNA. Using 0.02 μg of this poly(A)$^+$RNA, a cDNA was synthesized by means of RNA PCR Kit (Takara Shuzo, Co., Japan). The kind of primer used was a random 9mer and the total volume of the reaction mixture was 40 μl. As a negative control of cDNA synthesis, a reverse

transcriptase-free reaction mixture was also provided. First, the reaction mixture was incubated at 30℃ for 10 minutes to conduct an amplification reaction to some extent. Then, it was incubated at 42℃ for 30 minutes to let the reverse transcription reaction proceed. The enzyme was inactivated by heating at 99℃ for 5 minutes and the reaction system was cooled at 5℃ for 5 minutes.

After completion of the reverse transcription reaction, a portion of the reaction mixture was recovered and after dilution with distilled water, extraction was carried out with phenol/chloroform and further with diethyl ether. The extract was subjected to precipitation from ethanol and the precipitate was dissolved in a predetermined amount of distilled water for use as a cDNA sample. This cDNA solution and the plasmid DNA (pAKKO-19P2) were serially diluted and using primers specific to full-length receptor protein, PCR was carried out. The sequences of the primers prepared according to the base sequence of the coding region of the full-length receptor protein were CTGACTTATTTTCTGGGCTGCCGC (SEQ ID NO:33) for 5' end and AACACCGACACATAGACGGTGACC (SEQ ID NO:34) for 3' end.

The PCR reaction was carried out in a total volume of 100μl using 1μM each of the primers, 0.5μl of Taq DNA polymerase (Takara Shuzo Co., Japan), the reaction buffer and dNTPs accompanying the enzyme, and 10μl of template DNA (cDNA or plasmid solution). First the reaction mixture was heat-treated at 94℃ for 2 minutes for sufficient denaturation of the template DNA and subjected to 25 cycles of 95℃ x 30 seconds, 65℃ x 30 seconds, and 72℃ x 60 seconds. After completion of the reaction, 10μl of the reaction mixture was subjected to agarose gel electrophoresis and the detection and quantitative comparison of amplification products were carried out. As a result, a PCR product of the size (400 bp) predictable from the sequence of the cDNA

coding for the full-length receptor protein was detected [Fig. 15]. In the lane of the PCR reaction mixture using the product of the reverse transcriptase-free transcription system as the template, no specific band was detected, thus extruding the possibility of its being a PCR product derived from the genomic DNA of CHO cells. Moreover, no specific band appeared in the lane of mock cells, either. Therefore, it was clear that the product was not derived from the mRNA initially expressed in CHO cells [Fig. 15].

[Example 11]

Detection of the activity to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells in a rat whole brain extract

A crude peptide fraction was prepared from rat whole brain by the following procedure. The rat whole brain enucleated immediately after sacrifice was frozen in liquefied nitrogen and stored at -80℃. The frozen rat whole brain, 20 g (the equivalent of 10 rats) was finely divided and boiled in 80 ml of distilled water for 10 minutes. After the boiled tissue was quenched on ice, 4.7 ml of acetic acid was added at a final concentration of 1.0 M and the mixture was homogenized using a Polytron (20,000 rpm, 6 min.). The homogenate was stirred overnight and then centrifuged (10,000 rpm, 20 min.) to separate the supernatant. The sediment was homogenized in 40 ml of 1.0 M acetic acid and centrifuged again to recover the supernatant. The supernatants were pooled, diluted in 3 volumes of acetone, allowed to stand on ice for 30 minutes, and centrifuged (10,000 rpm, 20 min.) to recover the supernatant. The recovered supernatant was evaporated to remove acetone. To the resulting acetone-free concentrate was added 2 volumes of 0.05% trifluoroacetic acid(TFA)/H_2O and the mixture was applied to a reversed-phase C18 column (Prep C^{18} 125Å,

Millipore). After application of the supernatant, the column was washed with 0.05% TFA/H_2O, and gradient elution was carried out with 10%, 20%, 30%, 40%, 50%, and 60% CH_3CN/0.05%TFA/H_2O. The fractions were respectively divided into 10 equal parts and lyophilized. The dried sample derived from one animal equivalent of rat whole brain was dissolved in 20 μl of dimethyl sulfoxide (DMSO) and suspended in 1 ml of Hank's balanced saline solution (HBSS) supplemented with 0.05% bovine serum albumin (BSA) to provide a crude peptide fraction.

The full-length receptor protein-expressed CHO cells and mock CHO cells were seeded in a 24-well plate, 0.5 x 10^5 cells/well, and cultured for 24 hours. Then, [^{3}H] arachidonic acid was added at a final concentration of 0.25 μ Ci/well. Sixteen (16) hours after addition of [^{3}H] arachidonic acid, the cells were rinsed with 0.05% BSA-HBSS and the above-mentioned crude peptide fraction was added, 400 μ l/well. The mixture was incubated at 37℃ for 30 minutes and a 300 μ l portion of the reaction mixture (400 μl) was added to 4 ml of a scintillator and the amount of [^{3}H] arachidonic acid metabolite released into the reaction mixture was determined with a scintillation counter. As a result, an arachidonic acid metabolite-releasing activity specific to the full-length receptor protein expressed CHO cells (CHO-19P2) was detected in the 30% CH_3CN fraction of the eluate [Fig. 16].

[Example 12]

Detection of the activity to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells in a bovine hypothalamus extract

A crude peptide fraction was prepared from 360 g (the equivalent of 1 animals) of bovine brain tissue including hypothalamus in the same manner as in Example 11. A dried peptide sample per 0.05 animal was

dissolved in 40 μl of DMSO and suspended in 2 ml of 0.05% BSA-HBSS and the detection of arachidonic acid metabolite-releasing activity was attempted in the same manner as in Example 11. As a result, the activity to specifically promote release of arachidonic acid metabolites from the CHO-19P2 cell line was detected in the fraction eluted with 30% CH_3CN from a C18 column to which the crude bovine hypothalamus peptide fraction had been applied [Fig. 17].

[Example 13]

Preparation of the activity (peptide) to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells by purification from bovine hypothalamus

A typical process for harvesting the activity to specifically promote release of arachidonic acid metabolites from the CHO-19P2 cell line by purification from bovine hypothalamus is now described. A frozen bovine brain tissue specimen including hypothalamus, 4.0 kg (the equivalent of 80 animals) was ground and boiled in 8.0 L of distilled water for 20 minutes. After quenching on ice, 540 ml of acetic acid was added at a final concentration of 1.0 M and the mixture was homogenized using a Polytron (10,000 rpm, 12 min.). The homogenate was stirred overnight and then centrifuged (9,500 rpm, 20 min) to recover a supernatant. The sediment was suspended in 4.0 L of 1.0 M acetic acid and homogenized with the Polytron and centrifuged again to recover a further supernatant. The supernatants were pooled and TFA was added at a final concentration of 0.05%. The mixture was applied to reversed-phase C18 (Prep C18 125Å, 160 ml; Millipore) packed in a glass column. After addition, the column was washed with 320 ml of 0.05% TFA/H_2O and 3-gradient elution was carried out with 10%, 30%, and 50% CH_3CN/0.05% TFA/H_2O. To the 30% CH_3CN/0.05% TFA/H_2O

fraction was added 2 volumes of 20 mM CH_3COONH_4/H_2O and the mixture was applied to the cation exchange column HiPrep CM-Sepharose FF (Pharmacia). After the column was washed with 20 mM CH_3COONH_4/10% CH_3CN/H_2O, 4-gradient elution was carried out with 100 mM, 200 mM, 500 mM, and 1000 mM CH_3COONH_4/10% CH_3CN/H_2O. In the 200 mM CH_3COONH_4 fraction, activity to specifically promote release of arachidonic acid metabolites from CHO-19P2 was detected. Therefore, this fraction was diluted with 3 volumes of acetone, centrifuged for deproteination, and concentrated in an evaporator. To the concentrated fraction was added TFA (final concentration 0.1%) and the mixture was adjusted to pH4 with acetic acid and applied to 3 ml of the reversed-phase column RESOURCE RPC (Pharmacia). Elution was carried out on a concentration gradient of 15%-30% CH_3CN. As a result, activity to specifically promote the release of arachidonic acid metabolites from the CHO-19P2 cell line was detected in the 19%-21% CH_3CN fraction. The active fraction eluted from RESOURCE RPC was lyophilized, dissolved with DMSO, suspended in 50 mM MES pH 5.0/10% CH_3CN, and added to 1 ml of the cation exchange column RESOURCE S. Elution was carried out on a concentration gradient of 0 M-0.7 M NaCl. As a result, the activity to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells was detected in the 0.32 M-0.46 M NaCl fraction. The active eluate from RESOURCE S was lyophilized, dissolved with DMSO, suspended in 0.1% TFA/H_2O, and added to reversed-phase column C18 218TP5415 (Vydac), and elution was carried out on a concentration gradient of 20%-30% CH_3CN. As a result, the activity to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells was detected in the three fractions 22.5%, 23%, and 23.5% CH_3CN (these active fractions are designated as P-1, P-2, and P-3)

[Fig. 18]. Of the three active fractions, the 23.5% CH_3CN fraction (P-3) was lyophilized, dissolved with DMSO, suspended in 0.1% TFA/H_2O, and added to the reversed-phase column diphenyl 219TP5415 (Vydac), and elution was carried out on a gradient of 22%-25% CH_3CN. As a result, the activity to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells was converged by recovered in one elution peak obtained with 23% CH_3CN [Fig. 19]. The peak activity fraction from the reverse-phased column diphenyl 219TP5415 was lyophilized, dissolved with DMSO, suspended in 0.1% TFA/H_2O, and added to the reversed-phase column μRPC C2/C18 SC 2.1/10 (Pharmacia), and elution was carried out on a gradient of 22%-23.5% CH_3CN. As a result, the activity to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells was detected in the two peaks eluted with 23.0% and 23.2% CH_3CN [Fig. 20].

[Example 14]

Determination of the amino acid sequence of the peptide having the activity to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells as purified from bovine hypothalamus

The amino acid sequence of the peptide (P-3) having activity to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells as purified in Example 13 was determined. The fraction of peak activity from the reversed-phase μRPC C2/C18 SC 2.1/10 was lyophilized and dissolved in 20μl of 70% CH_3CN and analyzed for amino acid sequence with the peptide sequencer (ABI.491). As a result, the sequence defined by SEQ ID NO:3 was obtained. However, the 7th and 19th amino acids were not determined by only the analysis of amino acid sequence.

[Example 15]

Preparation of the active substance (peptide) which specifically promotes release of arachidonic acid metabolites from CHO-19P2 cells as purified from bovine hypothalamus

Of the three active fractions obtained with Vydac C18 218TP5415 in Example 13, the active fraction (P-2) eluted with 23.0% CH_3CN was further purified. This active fraction was lyophilized, dissolved with DMSO, suspended in 0.1% TFA/distilled H_2O, and added to reversed-phase column diphenyl 219TP5415 (Vydac), and elution was carried out on a gradient of 21.0%-24.0% CH_3CN. As a result, activity to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells was detected in a peak eluted with 21.9% CH_3CN. This fraction was lyophilized, dissolved with DMSO, suspended in 0.1% TFA/distilled H_2O, and added to reversed-phase μRPC C2/C18 SC 2.1/10 (Pharmacia), and elution was carried out on a CH_3CN gradient of 21.5%-23.0%. As a result, the activity to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells converged in one peak eluted with 22.0% CH_3CN[Fig. 21].

[Example 16]

Determination of the amino acid sequence of the peptide (P-2) purified from bovine hypothalamus which specifically promotes release of arachidonic acid metabolites from CHO-19P2 cells

The amino acid sequence of the peptide (P-2) having the activity to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells as purified in Example 15 was determined. The peak activity fraction from the reversed-phase column μRPC C2/C18 SC 2.1/10 was lyophilized, dissolved in 20μl of 70% CH_3CN, and analyzed for amino acid sequence with the peptide sequencer (ABI, 492) (SEQ ID NO:4).

[Example 17]

Preparation of a poly(A)$^+$RNA fraction from bovine hypothalamus and synthesis of a cDNA

Using Isogen (Nippon Gene), total RNA was prepared from one animal equivalent of bovine hypothalamus. Then, using Fast Track (Invitrogen), a poly(A)$^+$RNA fraction was prepared. From 1 μg of this poly(A)$^+$RNA fraction, cDNA was synthesized using 3' RACE system (GIBCO BRL) and Marathon cDNA amplification kit (Clontech) according to the manuals and dissolved in 20 and 10 μl, respectively.

[Example 18]

Acquisition of cDNA coding for the amino acid sequence established in Example 14

To obtain a cDNA coding for a polypeptide comprising the amino acid sequence established in Example 14, the acquisition of a base sequence coding for SEQ ID NO:1 was attempted in the first place. Thus, primers P5-1 (SEQ ID NO:35), P3-1 (SEQ ID NO:36), and P3-2 (SEQ ID NO:37) were synthesized. (In the Sequence Table, I represents inosine). Using 0.5 μl of the cDNA prepared by 3' RACE in Example 17 as a template and EXTaq (Takara Shuzo Co., Japan) as DNA polymerase, 2.5 μl of accompanying buffer, 200 μM of accompanying dNTP, and primers P5-1 and P3-1 were added each at a final concentration of 200 nM, with water added to make 25 μl, and after one minute at 94℃, the cycle of 98℃ x 10 seconds, 50℃ x 30 seconds, 68℃ x 10 seconds was repeated 30 times. This reaction mixture was diluted 50-fold with tricine-EDTA buffer and using 2.5 μl of the dilution as a template and the primer combination of P5-1 and P3-2, the reaction was carried out in otherwise the same manner as described above. As the thermal cycler, Gene Amp 9600 (Perkin Elmer) was used. The amplification product was subjected to 4% agarose electrophoresis and ethidium bromide staining and a band of about 70 bp was cut out and subjected to

thermal fusion, phenol extraction, and ethanol precipitation. The recovered DNA was subcloned into plasmid vector PCRTMII according to the manual of TA Cloning kit (Invitrogen). The vector was then introduced into E. coli JM109 and the resultant transformant was cultured in ampicillin-containing LB medium. The plasmid obtained with an automatic plasmid extractor (Kurabo) was reacted according to the manual of Dye Terminator Cycle Sequencing Kit (ABI) and decoded with a fluorescent automatic DNA sequencer (ABI). As a result, the sequence shown in Fig. 22 was obtained and confirmed to be part of the base sequence coding for SEQ ID NO:1.

[Example 19]

Acquisition of a bioactive polypeptide cDNA by RACE using the sequence established in Example 18

First, for amplification (5' RACE) of the sequence at 5' end, the two primers PE (SEQ ID NO:38) and PDN (SEQ ID NO:39) were synthesized by utilizing the sequence shown in Fig. 22. The cDNA prepared using Marathon cDNA amplification kit in Example 17 was diluted 100-fold with tricine-EDTA buffer. Then, in the same manner as Example 2, a reaction mixture was prepared using 2.5 μl of the dilution and a combination of the adapter primer AP1 accompanying the kit and the primer PE and after one minute at 94℃, the cycle of 98℃ x 10 seconds and 68℃ x 5 minutes was repeated 30 times. This reaction system was further diluted 50-fold with tricine-EDTA buffer and using 2.5 μl of the dilution as a template and the changed primer combination of AP1 and PDN, the reaction was conducted at 94℃ for one minute, followed by 4 cycles of 94℃ x 1 minute, 98℃ x 10 seconds, 72℃ x 5 minutes, 4 cycles of 98℃ x 10 seconds, 70℃ x 5 minutes, and 26 cycles of 98℃ x 10 seconds, 68℃ x 5 minutes. The amplification product was electrophoresed on 1.2% agarose gel and

stained with ethidium bromide and a band of about 150 bp was cut out and centrifugally filtered through a centrifugal filter tube (Millipore), extracted with phenol, and precipitated from ethanol. The recovered DNA was subcloned into plasmid vector PCR™II according to the manual of TA Cloning Kit (Invitrogen). The vector was then introduced into E. coli JM109 and the resulting transformant was cultured and the sequence of the inserted cDNA fragment was analyzed as in Example 18. As a result, the sequence shown in Fig. 23 was obtained. Based on this sequence, primers FB (SEQ ID NO:40) and FG (SEQ ID NO:41) were synthesized and the 3' sequence was cloned (3' RACE). Using the same template as that for 5' RACE in the same quantity and the combination of the accompanying adapter primer AP1 with the primer FC, PCR was carried out at 94℃ for 1 minute, followed by 5 cycles of 98℃ x 10 seconds, 72℃ x 5 minutes, 5 cycles of 98℃ x 10 seconds, 70℃ x 5 minutes, and 25 cycles of 98℃ x 10 seconds, 68℃ x 5 minutes. Then, using 2.5μl of a 50- fold dilution of this reaction mixture in tricine-EDTA buffer as the template and the combination of the accompanying primer AP2 with the primer FB, the reaction was further conducted at 94℃ for one minute, followed by 4 cycles of 98℃ x 10 seconds, 72℃ x 5 minutes, 4 cycles of 98℃ x 10 seconds, 70℃ x 5 minutes, and 27 cycles of 98℃ x 10 seconds, 68℃ x 5 minutes. The amplification product was electrophoresed on 1.2% agarose gel and stained with ethidium bromide and a band of about 400 bp was cut out and the DNA was recovered as in 5'-RACE. This DNA fragment was subcloned into plasmid vector pCRTMII and introduced into E. coli JM109 and the sequence of the inserted cDNA fragment in the resulting transformant was analyzed. From the results of 5' RACE and 3' RACE, the DNA sequence [Fig. 24] coding for the complete coding region of the bioactive polypeptide

defined by SEQ ID NO:1 was established. Thus, in Fig. 24 (a) and (b), the base134 is G, the base184 is T or C, and the base245 was T or C.

The cDNA shown in Fig. 24 was the cDNA encoding a polypeptide consisting of 98 amino acids. The fact that the amino acids in 1 - 22-positions comprise a cluster of hydrophobic amino acids taken together with the fact that the N-terminal region of the active peptide begins with Ser in 23-position as shown in Example 14 suggested that the amino acids 1-22 represent a secretion signal sequence. On the other hand, the Gly-Arg-Arg-Arg sequence in 54-57 positions of the polypeptide was found to be a typical amino acid sequence motif which exists in the event of cleavage of a bioactive peptide. As it is the case with this cleavage motif, it is known that because of the presence of Gly, the C-terminus of the product peptide is frequently amidated.

The P-3 N-terminal sequence data of Example 14 and P-2 N-terminal sequence data in Example 16 coupled with this GlyArgArgArg sequence suggest that at least same of the bioactive peptides cut out from the polypeptide encoded by this cDNA are defined by SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9 or SEQ ID NO:10.

[Example 20]

Acquisition of a DNA fragment comprising the full coding region of bovine-derived bioactive polypeptide cDNA by PCR

Using the cDNA prepared with Marathon cDNA amplification kit in Example 17 as a template, a DNA fragment including the entire coding region of bioactive polypeptide cDNA was constructed. First, based on the sequence of cDNA elucidated in Example 19, two primers having base sequences defined by SEQ ID NO:42 and SEQ ID NO:43, respectively, were synthesized.

BOVF

5'-GTGTCGACGAATGAAGGCGGTGGGGGCCTGGC-3' (SEQ ID NO:42)

BOVR (24 mer)

5'-AGGCTCCCGCTGTTATTCCTGGAC-3' (SEQ ID NO:43)

BOVF contains the initiation codon of bioactive polypeptide cDNA and is a sense sequence corresponding to -2 - +22 (A of the initiation codon ATG being reckoned as +1) with restriction enzyme SalI site added. On the other hand, BOVR is an antisense sequence corresponding to +285 - +309 which includes the termination codon of bioactive polypeptide cDNA.

The PCR was conduced as follows. The cDNA prepared using Marathon cDNA amplification kit in Example 17 was diluted 100-fold in tricine-EDTA buffer and using 2.5μl of the dilution, a reaction mixture was prepared as in Example 2 and subjected to 94℃ x 1 minute, 3 cycles of 98℃ x 10 seconds, 72℃ x 5 minutes, 3 cycles of 98℃ x 10 seconds, 70℃ x 5 minutes, and 27 cycles of 98℃ x 10 seconds, 68℃ x 5 minutes. The amplification product was subjected to 2% agarose electrophoresis and ethidium bromide staining and a band of about 320 bp was cut out. The DNA was recovered and subcloned in plasmid vector pCRTMII as in Example 3. The vector was introduced into Escherichia coli JM109 to provide the transformant E. coli JM109/pBOV3. The sequence of the cDNA fragment inserted in the transformant was then analyzed. As a result, this DNA fragment was confirmed to be a fragment covering the entire coding region of the bioactive polypeptide cDNA.

[Example 21]

Synthesis of Ser-Arg-Ala-His-Gln-His-Ser-Met-Glu-Ile-Arg-Thr-Pro-Asp-Ile-Asn-Pro-Ala-Trp-Tyr-Ala-Gly-Arg-Gly-Ile-Arg-Pro-Val-Gly-Arg-Phe-NH_2 (19P2-L31)

1) Synthesis of Ser(Bzl)-Arg(Tos)-Ala-His(Bom)-Gln-

His(Bom)-Ser(Bzl)-Met-Glu(OcHex)-Ile-Arg(Tos)-Thr(Bzl)-Pro-Asp(OcHex)-Ile-Asn-Pro-Ala-Trp(CHO)-Tyr(Br-Z)-Ala-Gly-Arg(Tos)-Gly-Ile-Arg(Tos)-Pro-Val-Gly-Arg(Tos)-Phe-pMBHA-resin

The reactor of a peptide synthesizer (Applied Biosystems 430A) was charged with 0.71 g (0.5 mmole) of commercial p-methyl-BHA resin (Applied Biosystems, currently Perkin Elmer). After wetting with DCM, the initial amino acid Boc-Phe was activated by the HOBt/DCC method and introduced into the p-methyl-BHA resin. The resin was treated with 50% TFA/DCM to remove Boc and make the amino group free and neutralized with DIEA. To this amino group was condensed the next amino acid Boc-Arg (Tos) by the HOBt/DCC method. After the absence of unreacted amino function was verified by ninhydrin test, a sequential condensation of Boc-Gly, Boc-Val, Boc-Pro, Boc-Arg(Tos), Boc-Ile, Boc-Gly, Boc-Arg(Tos), Boc-Gly, Boc-Ala, Boc-Tyr(Br-Z) was carried out. The Boc-Ala, Boc-Tyr (Br-Z), the condensation of which was found insufficient by ninhydrin test, was recondensed to complete the reaction. The resin was dried and a half of the resin was withdrawn. To the remainder, Boc-Trp(CHO), Boc-Ala, Boc-Pro, Boc-Asn, Boc-Ile, Boc-Asp(OcHex), Boc-Pro, Boc-Thr(Bzl), Boc-Arg(Tos), Boc-Ile, Boc-Glu(OcHex), Boc-Met, Boc-Ser(Bzl), Boc-His(Bom), Boc-Gln, Boc-His(Bom), Boc-Ala, Boc-Arg(Tos), Boc-Ser(Bzl) were serially condensed and recondensed until sufficient condensation was confirmed by ninhydrin test. After introduction of the full sequence of amino acids of 19P2-L31, the resin was treated with 50% TFA/DCM to remove Boc groups on the resin and, then, dried to provide 1.28 g of the peptide resin.

2) Synthesis of Ser-Arg-Ala-His-Gln-His-Ser-Met-Glu-Ile-Arg-Thr-Pro-Asp-Ile-Asn-Pro-Ala-Trp-Tyr-Ala-Gly-

Arg-Gly-Ile-Arg-Pro-Val-Gly-Arg-Phe-NH_2(19P2-L31)

In a Teflon hydrogen fluoride reactor, the resin obtained in 1) was reacted with 3.8 g of p-cresol, 1 ml of 1,4-butanedithiol, and 10 ml of hydrogen fluoride at 0℃ for 60 minutes. The hydrogen fluoride and 1,4-butanedithiol (1 ml) were distilled off under reduced pressure and the residue was diluted with 100 ml of diethyl ether, stirred, filtered through a glass filter, and the fraction on the filter was dried. This fraction was suspended in 50 ml of 50% acetic acid/H_2O and stirred to extract the peptide. After separation of the resin, the extract was concentrated under reduced pressure to about 5 ml and chromatographed on Sephadex G-25 (2 x 90 cm). Development was carried out with 50% acetic acid/ H_2O and the 114 ml - 181 ml fraction was pooled and lyophilized to recover 290 mg of white powders containing 19P2-L31. The powders were applied to a reversed-phase column of LiChroprep RP-18 (Merck) and repeatedly purified by gradient elution using 0.1% TFA/ H_2O and 0.1% TFA-containing 30% acetonitrile/ H_2O. The fraction eluted at about 25% acetonitrile was pooled and lyophilized to provide 71 mg of white powders.

Mass spectrum $(M+H)^+$ 3574.645

HPLC elution time 18.2 min.

Column conditions

Column: Wakosil 5C18 (4.6 x 100 mm)

Eluent: A (0.1% TFA/ H_2O)

B (0.1% TFA-containing 50 % acetonitrile/ H_2O)

Linear gradient elution from A to B (25 min.)

Flow rate: 1.0 ml/min.

[Example 22]

Synthesis of Ser-Arg-Ala-His-Gln-His-Ser-Met(O)-Glu-Ile-Arg-Thr-Pro-Asp-Ile-Asn-Pro-Ala-Trp-Tyr-Ala-Gly-Arg-Gly-Ile-Arg-Pro-Val-Gly-Arg-Phe-NH_2(19P2-L31(O))

In 20 ml of 5% acetic acid/ H_2O was dissolved 6 mg of synthetic 19P2-L31 and the Met only was selectively oxidized with 40 μl of 30% H_2O. After completion of the reaction, the reaction mixture was immediately applied to a reversed-phase column of LiChroprep RP-18 (Merck) for purification to provide 5.8 mg of the objective peptide.

Mass spectrum $(M+H)^+$ 3590.531

HPLC elution time 17.9 min.

Column conditions

Column: Wakosil 5C18 (4.6 x 100 mm)

Eluent: A (0.1% TFA/ H_2O)

B (0.1% TFA-containing 50% acetonitrile/ H_2O)

Linear gradient elution from A to B (25 min.)

Flow rate: 1.0 ml/min.

[Example 23]

Synthesis of Thr-Pro-Asp-Ile-Asn-Pro-Ala-Trp-Tyr-Ala-Gly-Arg-Gly-Ile-Arg-Pro-Val-Gly-Arg-Phe-NH_2(19P2-L20)

To the resin subjected to condensations up to Boc-Tyr(Br-Z) in Example 21-1) was further condensed Boc-Trp(CHO), Boc-Ala, Boc-Pro, Boc-Asn, Boc-Ile, Boc-Asp(OcHex), Boc-Pro, Boc-Thr(Bzl) serially in the same manner to provide 1.14 g of Boc-Thr(Bzl)-Pro-Asp(OcHex)-Ile-Asn-Pro-Ala-Trp(CHO)-Tyr(Br-Z)-Ala-Gly-Arg(Tos)-Gly-Ile-Arg(Tos)-Pro-Val-Gly-Arg(Tos)-Phe-pMBHA-resin. This resin was treated with hydrogen fluoride and columnwise purified in the same manner as Example 21-2) to provide 60 mg of white powders.

Mass spectrum $(M+H)^+$ 2242.149

HPLC elution time 10.4 min.

Column conditions

Column: Wakosil 5C18 (4.6 x 100 mm)

Eluent: A (0.1% TFA-containing 15% acetonitrile/ H_2O)

B (0.1% TFA-containing 45% aceto

nitrile/ H_2O)

Linear gradient elution from A to B (15 min.)

Flow rate: 1.0 ml/min.

[Example 24]

Determination of arachidonic acid metabolites-releasing activity of synthetic peptide (19P2-L31)

The activity of the peptide (19P2-L31) synthesized in Example 21 to specifically release arachidonic acid metabolites from CHO-19P2 cells was assayed in the same manner as Example 11. The synthetic peptide was dissolved in degassed distilled H_2O at a concentration of 10^{-3}M
and diluted with 0.05% BSA-HBSS and the activity to promote release of arachidonic acid metabolites from CHO-19P2 cells at each concentration was assayed using the amount of [^{3}H]arachidonic acid metabolites as the indicator. As a result, concentration-dependent arachidonic acid metabolite-releasing activity was detected over the range of 10^{-12}M - 10^{-6}M [Fig. 25]. When the arachidonic acid metabolite-releasing activity of peptide 19P2-L31(O), i.e. the methionine-oxidation product of 19P2-L31 synthesized in Example 22, was compared with that of 19P2-L31, it was found that the activity of 19P2-L31(O) was equivalent to the activity of 19P2-L31 as can be seen from Fig. 26.

[Example 25]

Determination of arachidonic acid metabolites-releasing activity of synthetic peptide (19P2-L20)

The activity of the synthetic equivalent (19P2-L20) of natural peptide P-2 as synthesized in Example 23 to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells was determined as in Example 11. Thus, the synthetic peptide was dissolved in degassed distilled H_2O at a final concentration of 10^{-3}M and this solution was serially diluted with 0.05% BAS-HBSS.

The activity to specifically promote release of arachidonic acid metabolites from CHO-19P2 cells at each concentration was assayed using the amount of [^{3}H]arachidonic acid metabolites as the indicator.

As a result, concentration-dependent arachidonic acid metabolite-releasing activity was detected over the range of 10^{-12} - 10^{-6}M in nearly the same degree as 19P2-L31 [Fig. 27].

[Example 26]

Analysis of the coding region base sequence of bovine genomic DNA

pBOV3 was digested with restirction enzyme EcoRI and after fractionation by agarose gel electrophoresis, the DNA corressponding to the cDNA fragment was recovered to prepare a probe. This DNA was labeled with 32P using a multiprime DNA labeling kit (Amersham). About 2.0×10^6 phages of Bovine Genomic Library (Clontech BL1015j) constructed using cloning vector EMBL3 SP6/T7 and Escherichia coli K802 as the host were seeded in an LB agar plate and cultured overnight for plaque formation. The plaques were transferred to a nitrocellulose filter and after alkaline modification and neutralization, heat-treated (80℃, 2 hours) to inactivate the DNA. This filter was incubated with the labeled probe in 50% formamide-Hybri buffer (50% formamide, 5 x Denhardt solution, 4 x SSPE, 0.1 mg/ml heat-denatured salmon sperm DNA, 0.1% SDS) at 42℃ overnight for hybridization. After this hybridization, the filter was washed with 2 x SSC, 0.1% SDS at room temperature for 1.5 hours, and further washed in the same buffer at 55℃ for 30 minutes. Detection of the clone hybridizing with the probe was carried out on Kodak X-ray film (X-OMATTMAR) after 4 days of exposure using a sensitization screen at -80℃. After development of the film, the film was collated with plate positions and the phages which had

hybridized were recovered. Then, plating and hybridization were repeated in the same manner for cloning of the pharges.

The cloned phages were prepared on a large scale by the plate lysate method and the phage DNA was extracted. Then, cleavage at the restriction enzyme SalI and BamHI cleavage sites at both ends of the cloning site of the vector and detection of the inserted fragment derived from bovine genomic DNA was carried out by 1.2% agarose gel electrophoresis [Fig. 28]. As a result, in the case of BamHI digestion, 3 fragments were detected in addition to the bands derived from the phages. In the case of SalI digestion, one band overlapping the phage band was detected. The SalI-digested fragment being considered to harbor the full length and in order to subclone this fragment into a plasmid vector, it was ligated to BAP (E. coli-derived alkaline phosphatase)-treated plasmid vector pUC18 (Pharmacia) and introduced into E. coli JM109. From this microorganism, a genome-derived SalI fragment-inserted plasmid DNA was prepared on a production scale and the base sequence in the neighborhood of its coding region was analyzed using Perkin Elmer Applied Biosystems 370A fluorecent sequencer and the same manufacturer's kit. As a result, the sequence shown in Fig. 29 was obtained. Comparison with the coding region of cDNA reveals that because of its being derived from genomic DNA, the coding region is divided in two by a 472 bp intron [Fig. 30]. Fig. 31 and SEQ ID NO:44 present the amino acid sequence predicted from this bovine genome coding region (excluding the intron region).

[Example 27]

Preparation of rat medulla oblongata poly(A)$^+$RNA fraction and synthesis of cDNA

Using Isogen (Nippon Gene), total RNA was prepared

from the dorsal region of rat medulla oblongata and using FastTrack (Invitrogen), poly(A)$^+$RNA fraction was prepared. To 5μg of this poly(A)$^+$RNA was added the primer random DNA hexamer (BRL) and using Moloney mouse leukemia reverse transcriptase (BRL) and the accompanying buffer, complementary DNA was synthesized. The reaction product was precipitated from ethanol and dissolved in 12μl of DW. In addition, from 1μg of this poly(A)$^+$RNA, a cDNA was synthesized using Marathon cDNA amplification kit (Clontech) according to the manual and dissolved in 10μl of DW.

[Example 28]

Acquisition of rat bioactive polypeptide cDNA by RACE

To obtain the full coding region of rat bioactive polypeptide cDNA, an experiment was performed in the same manner as the acquisition of bovine cDNA. First, PCR was carried out using the same primers P5-1 (SEQ ID NO:35) and P3-1 (SEQ ID NO:36) as used in Example 18 as primers and the complementary DNA synthesized in Example 27 using the primer random DNA hexamer (BRL) and Moloney mouse leukemia reverse transcriptase (BRL) as a template. The reaction system was composed of 1.25μl of the template cDNA, 200μM of dNTP, 1μM each of the primers, ExTaq (Takara Shuzo Co., Japan) as DNA polymerase, and 2.5μl of the accompanying buffer, with a sufficient amount of water to make a total of 25μl. The reaction was carried out at 94℃ for 1 minute, followed by 40 cycles of 98℃ x 10 seconds, 50℃ x 30 seconds, and 72℃ x 5 seconds, and the reaction mixture was then allowed to stand at 72℃ for 20 seconds. The thermal cycler used was GeneAmp2400 (Perkin Elmer). The amplification product was subjected to 4% agarose electrophoresis and ethidium bromide staining and the band of about 80 bp was cut out. Then, in the manner described in Example 19, the DNA was recovered,

subcloned into plasmid vector pCRTMII, and introduced into E. coli JM109, and the inserted cDNA fragment was sequenced. As a result, a partial sequence of rat bioactive polypeptide could be obtained. Based on this sequence, two primers, namely RA (SEQ ID NO:75) for 3' RACE and RC (SEQ ID NO:76) for 5' RACE were synthesized and 5' and 3' RACEs were carried out.

RA:5'-CARCAYTCCATGGAGACAAGAACCCC-3'
(where R means A or G; Y means T or G) (SEQ ID NO:75)

RC:5'-TACCAGGCAGGATTGATACAGGGG-3'
(SEQ ID NO:76)

As a template, the template synthesized using Marathon cDNA amplification kit (Clontech) in Example 27 was diluted 40-fold with the accompanying tricine-EDTA buffer and 2.5μl of the dilution was used. As primers, RA and the adapter primer AP1 accompanying the kit were used for 3' RACE, and RC and AP1 for 5' RACE. The reaction mixture was prepared in the same manner as above. The reaction conditions were 94℃ x 1 minute, 5 cycles of 98℃ x 10 seconds, 72℃ x 45 seconds, 3 cycles of 98℃ x 10 seconds, 70℃ x 45 seconds, and 40 cycles of 98℃ x 10 seconds, 68℃ x 45 seconds. As a result, a band of about 400 bp was obtained from 3' RACE and bands of about 400 bp and 250 bp from 5' RACE. These bands were recovered in the same manner as above and using them as templates and the primers used in the reaction, sequencing was carried out with Dye Terminator Cycle Sequencing Kit (ABI). As a result, the sequence up to poly A could be obtained from the region considered to be the 5' noncoding region.

[Example 29]

Acquisition of the full-length cDNA of rat bioactive polypeptide by PCR

Based on the sequence obtained in Example 28, two primers, viz. rF for the region including the

initiation codon (SEQ ID NO:77) and rR for the 3' side from the termination codon (SEQ ID NO:78), were synthesized to amplify the fragment including the full-length cDNA.

rF:5'-GGCATCATCCAGGAAGACGGAGCAT-3' (SEQ ID NO:77)

rR:5'-AGCAGAGGAGAGGGAGGGTAGAGGA-3' (SEQ ID NO:78)

Using the cDNA prepared using Moloney mouse leukemia reverse transcriptase in Example 27 as a template and ExTaq (Takara Shuzo Co., Japan), PCR was carried out by repeating 40 cycles of 95℃ x 30 seconds, 68℃ x 60 seconds. The amplification product was subjected to agarose electrophoresis and ethidium bromide staining and a band of about 350 bp was cut out. The DNA was recovered, subcloned into plasmid vector pCRTMII, and introduced into E. coli JM109 as in Example 19. The plasmid was extracted from the transformant and the base sequence was determined. As a result, E. coli JM 109/pRAV3 having the full-length cDNA of rat bioactive polypeptide was obtained [Fig. 32].

[Example 30]

Synthesis of cDNA from the human total brain ply(A)$^+$RNA fraction

From 1 μg of human total brain poly(A)$^+$RNA fraction (Clontech), cDNA was synthesized with Marathon cDNA amplification kit (Clontech) according to the manual and dissolved in 10μl. In addition, the random DNA hexamer (BRL) was added as primer to 5μg of the same poly(A)$^+$RNA fraction and using Moloney mouse leukemia reverse transcriptase (BRL) and the accompanying buffer, complementary DNA was synthesized. The reaction product was precipitated from ethanol and dissolved in 30μl of TE.

[Example 31]

Acquisition of human bioactive polypeptide cDNA by RACE

From the amino acid sequence of rat bioactive polypeptide established in Example 28 [Fig. 33], the well-preserved regions of rat and bovine polypeptides were selected and the following 3 primers R1 (SEQ ID NO:79), R3 (SEQ ID NO:80), and R4 (SEQ ID NO:81) were synthesized. Then, amplification of the region flanked by them was attempted by PCR using human cDNA as a template. Referring to Fig. 33, bovine. aa represents the amino acid sequence of bovine polypeptide, bovine. seq represents the base sequence of the DNA coding for bovine polypeptide, and rat. seq represents the base sequence of the DNA coding for rat polypeptide.

R1:5'-ACGTGGCTTCTGTGCTTGCTGC-3' (SEQ ID NO:79)

R3:5'-GCCTGATCCCGCGGCCCGTGTACCA-3' (SEQ ID NO:80)

R4:5'-TTGCCCTTCTCCTGCCGAAGCGGCCC-3' (SEQ ID NO:81)

The cDNA prepared using Marathon cDNA amplification kit (Clontech) in Example 30 was diluted 30-fold with tricine-EDTA buffer and 0.25μl of the dilution was used as a template. The reaction mixture was composed of 200μM of dNTP, 0.2μM each of the primers R1 and R4, a 50:50 mixture of Taq Start Antibody (Clontech) and DNA polymerase ExTaq (Takara Shuzo Co., Japan), 2.5μl of the accompanying buffer, and a sufficient amount of water to make a total of 25 μl. The reaction conditions were 94℃ x 1 minute, followed by 42 cycles of 98℃ x 10 seconds, 68℃ x 40 seconds, and 1 minute of standing at 72℃. Then, using 1μl of a 100-fold dilution of the above reaction mixture in tricine-EDTA buffer as a template, the same reaction mixture as above except that the primer combination was changed to R1 and R3 was prepared and PCR was carried out in the sequence of 94℃ x 1 minute and 25 cycles of 98℃ x 10 seconds, 68℃ x 40 seconds. The amplification product was subjected to 4% agarose electrophoresis and ethidium bromide staining. As a result, a band of about 130 bp was obtained as

expected. This band was recovered in the same manner as in Example 28 and using the recovered fragment as a template, sequencing was carried out with Dye Terminator Cycle Sequencing Kit (ABI). As a result, a partial sequence of human bioactive polypeptide could be obtained. Therefore, based on this sequence, primers HA (SEQ ID NO:82) and HB (SEQ ID NO:83) were synthesized for 3' RACE and primers HE (SEQ ID NO:84) and HF (SEQ ID NO:85) for 5' RACE and 5' and 3' RACEs were carried out.

HA:5'-GGCGGGGGCTGCAAGTCGTACCCATCG-3' (SEQ ID NO:82)
HB:5'-CGGCACTCCATGGAGATCCGCACCCCT-3' (SEQ ID NO:83)
HE:5'-CAGGCAGGATTGATGTCAGGGGTGCGG-3' (SEQ ID NO:84)
HF:5'-CATGGAGTGCCGATGGGTACGACTTGC-3' (SEQ ID NO:85)

As the template, 2.5μl of a 20-fold dilution of the cDNA prepared in Example 30 in tricine-EDTA buffer was used. For the initial PCR, reaction mixtures were prepared in the same manner as above except that HA and adapter primer AP1 were used for 3' RACE and HE and AP1 for 5' RACE. The reaction sequence was 94℃ x 1 minute, 5 cycles of 98℃ x 10 seconds, 72℃ for 35 seconds, 5 cycles of 98℃ x 10 seconds, 70℃ x 35 seconds, and 40 cycles of 98℃ x 10 seconds, 68℃ x 35 seconds. Then, using 1μl of a 100-fold dilution of this reaction mixture in tricine-EDTA buffer as a template, a second PCR was carried out in the same cycles as the first PCR. However, the reaction mixture was prepared using primers HB and AP1 for 3' RACE or HF and AP2 for 5' RACE and Klen Taq (Clontech) as DNA polymerase and the accompanying buffer. As a result, a band of about 250 bp was obtained from 3' RACE and a band of about 150 bp from 5'-RACE. These bands were sequenced by the same procedure as above and using them in combination with the partial sequence obtained previously, the sequence from the region presumed to be 5'-noncoding region to polyA of human bioactive

polypeptide was obtained.

[Example 32]

Acquisition of human bioactive polypeptide full-length cDNA by PCR

Based on the sequence obtained in Example 31, two primers 5H (SEQ ID NO:86) and 3HN (SEQ ID NO:87) were synthesized for amplification of a fragment including full-length cDNA.

5H:5'-GGCCTCCTCGGAGGAGCCAAGGGATGA-3' (SEQ ID NO:86)

3HN:5'-GGGAAAGGAGCCCGAAGGAGAGGAGAG-3' (SEQ ID NO:87)

Using 2.5μl of the cDNA prepared using Moloney mouse leukemia reverse transcriptase (BRL) in Example 30 as a template and the reaction mixture prepared using Klen Taq DNA polymerase (Clontech), the PCR reaction was conducted in the sequence of 94℃ x 1 minute and 40 cycles of 98℃ x 10 seconds, 68℃ x 30 seconds. The fragment of about 360 bp obtained was recovered and subcloned (pCR™ 2.1 was used as the vector) in otherwise the same manner as Example 29. The plasmid was recovered and its base sequence was determined. As a result, E. coli JM109/pHOV7 harboring the human bioactive polypeptide full-length cDNA was obtained [Fig. 34]. In regard to the amino acid sequence of the translation region, a comparison was made between this human bioactive polypeptide and the bovine polypeptide shown in Example 20 or the rat polypeptide in Example 29 [Fig. 35].

[Example 33]

(1) Preparation of UHR-1 expression CHO cells

Recently, the orphan receptor UHR-1 has been cloned from the rat suprachiasmatic nucleus by Susan K. Welch and coworkers (Biochemical and Biophysical Research Communications, Vol. 209, No. 2, pp. 606-613, 1995).

Based on this report, the inventors of the present invention compared the amino acid sequence of

the protein encoded by the UHR-1 gene with the amino acid sequence of the protein encoded by hGR3.

As a result, the two sequences had 91.6% identity over 359 amino acids, suggesting that UHR-1 is a phGR3 homolog. In order to confirm that the protein encoded by UHR-1 functions as a receptor for 19P2-L31, the inventors of the present invention carried out a cloning of UHR-1 cDNA and subcloned it into CHO cells to construct a stable expression cell line as described below.

By the extraction using FastTrack™ Kit (Invitrogen), poly(A)$^+$RNA was prepared from the anterior lobe of the rat hypophysis. Then, using 0.2 μg of the poly(A)$^+$RNA as a template, a cDNA was synthesized on a total reaction scale of 40 μl using TaKaRa RNA PCR Kit (Takara Shuzo). The reaction product was extracted with phenol-chloroform (1:1), precipitated with ethanol, and dissolved in 10 μl of distilled water. Based on the known nucleotide sequence of rat UHR-1 cDNA (GenBank, Accession Number S77867), the following two synthetic DNA primers were prepared.

(1) 5'-GTTCACAG(GTCGAC)ATGACCTCAC-3'
(SalI recognition sequence in parentheses) (SEQ ID NO:95)

(2) 5'-CTCAGA(GCTAGC)AGAGTGTCATCAG-3'
(NheI recognition sequence in parentheses) (SEQ ID NO:96)

Using the above pair of primers (1) and (2) and the cDNA synthesized by the procedure described above as the template, a PCR was carried out. For this reaction, 5 μl of a 5-fold dilution of the cDNA solution, 1 μl of a 1:1 mixture of Ex Taq (Takara Shuzo) and Taq Start Antibody (Clontech), 5 μl of 10 x reaction buffer attached to Ex Taq, 4 μl of dNTP, and 1 μl each of the primers of 50 μM concentration were

used and the whole amount was made up to 50 μl with distilled water.

The PCR was performed according to the schedule of denaturing at 95℃ x 2 min. and 27 cycles each consisting of 95℃ x 30 sec., 65℃ x 30 sec. and 72℃, 1 min., followed by final extension at 72℃ x 7 min. After completion of cycling, a portion of the reaction mixture was electrophoresed on an agarose gel. After ethidium bromide staining, a 1.1 kbp (approx.) band was excised, centrifugally filtered using a centrifugal filtration tube (Millipore), extracted with phenol, and precipitated from ethanol to recover the DNA. The recovered DNA was subcloned into the plasmid vector pCR™II according to the manual of TA Cloning Kit (Invitrogen) (pCRII-UHR-1) and introduced into Escherichia coli JM109. The resultant transformant was cultured in ampicillin-containing LB medium and the plasmid was extracted with an automatic plasmid extractor (Kurabo).

This plasmid was subjected to sequencing reaction using ABI PRISM Dye Teriminator Cycle Sequencing Kit, FS (Perkin-Elmer) according to the manual and the nucleotide sequence was read out using a fluorescent automatated DNA sequencer (ABI).

The above sequencing revealed that the cDNA fragment obtained by PCR was a 1116bp fragment [Fig. 52]. Fig. 52 shows the nucleotide sequence of the full coding region of the rat UHR-1 constructed on the expression vector pAKKO-UHR-1 and the amino acid sequence encoded thereby. In Fig. 52, the underscored sequences (1) and (2) correspond to portions of the respective primer sequences. The bases different from those of the known nucleotide sequence (C in 664-position, G in 865-position, G in 897-position) are double-scored. The known nucleotide sequence presented here is a reproduction of GenBank Accession No. S77867.

One of those base substitutions involves an amino acid substitution of ^{289}Leu (CTC) → ^{289}Val (GTC). The construction of the UHR-1 expression vector was carried out as follows.

The pCRII-UHR-1 was cleaved with the restriction enzymes NheI (Takara Shuzo) and SalI (Takara Shuzo). The sample available on cleavage was electrophoresed on an agarose gel and stained with ethidium bromide, and the gel portion corresponding to the band was cut out. This gel fragment was put in a centrifuge tube with a filter (Millipore), frozen in a freezer, and thawed at room temperature. The tube was then centrifuged at 8000 rpm for 1 minute, whereupon a solution containing the DNA fragment was eluted out in the bottom of the filter. This solution was extracted with phenol, phenol-chloroform (1:1), and diethyl ether in the routine manner to remove impurities and the DNA was precipitated from ethanol to recover a cDNA fragment.

The pAKKO-111H was cleaved with the restriction enzymes NheI (Takara Shuzo) and SalI (Takara Shuzo) and the vector was isolated and extracted from an agarose gel in the same manner as above. Using Ligation System (Takara Shuzo), the cDNA fragment obtained above was reacted with the restriction enzyme digest of pAKKO-111H at 16℃ for 30 minutes. Using a portion of this ligation product, Escherichia coli JM109 was transformed to construct a transformant, Escherichia coli JM109/pAKKO-UHR-1. This transformant was cultured overnight in 2 ml of ampicillin (50 μg/ml)-containing LB medium and using an automatic plasmid extractor (Kurabo), the plasmid DNA (pAKKO-UHR-1) was obtained. The nucleotide sequence of the cDNA fragment-PAKKO-111H ligation site was analyzed with a fluorescent sequencer to confirm completion of the construction of the expression vector pAKKO-UHR-1.

(2) Introduction of the UHR-1 expression vector into

CHO dhfr$^-$ cells

In a 10 cm-diameter tissue culture dish, $1x10^6$ CHO dhfr$^-$ cells were seeded and cultured for 24 hours. From 20 μg of the UHR-1 expression vector pAKKO-UHR-1 obtained in (1), a DNA-liposome complex was prepared using a liposome-mediated gene transfer kit (Gene Transfer, Nippon Gene). The medium was replaced with fresh one and the DNA-liposome complex was added and incubated overnight. The medium was replaced with fresh one again and further incubated for 1 day. After the medium was replaced with a transformant screening medium, the complex was incubated for 2 days. The cells were harvested from the dish by trypsin-EDTA treatment and recultured at a low cell density for an enhanced yield of the transformant. By the above procedure, a CHO-UHR-1 cell line capable of stable, high expression of UHR-1 could be cloned.

[Example 34]

^{125}I labeling of 19P2-L31 and a receptor-binding experiment using the labeled 19P2-L31

The radiolabeling of 19P2-L31 was carried out using [^{125}I]-Bolton-Hunter Reagent (NEN/DuPont; NEX-120). First, 200 μl of [^{125}I]-Bolton-Hunter Reagent (2200 Ci/mmol) was transferred to a 500 μl Eppendorf's tube and dried thoroughly with nitrogen gas. It was redissolved in 2 μl of acetonitrile and, then, 4 μl of 50 mM phosphate buffer (pH 8.0) and 4 μl of $3x10^{-4}$ M synthetic 19P2-L31 were added. After mixing, the reaction was carried out at room temperature for 40 minutes. The reaction was then stopped with 5 μl of 1.0 M glycine buffer and the whole reaction mixture was applied onto a reversed phased column (Tosoh; TSK gel ODS-80TMCTP) to separate [^{125}I]-labeled 19P2-L31 ([^{125}I]-19P2-L31). The fraction containing [^{125}I]-19P2-L31 was diluted with 2 volumes of 50 mM Tris-HCl (pH 7.5)-0.1% BSA-0.05% CHAPS, distributed in small aliquots, and

stored at -20℃.

The receptor binding experiment was performed using CHO-19P2-9, CHO-UHR-1, and mock CHO as receptor expression CHO cells. CHO-19P2-9 cells were obtained by picking up a CHO-19P2 cell clone showing particularly high activity to stimulate the release of arachidonic acid metabolites by 19P2L-L31 from a limiting dilution culture in a 96-well microtiter plate. The mock CHO cells were control cells obtained by transformation with the expression vector pAKKO alone. Those cells, grown in tissue culture flasks, were respectively scraped off with 5 mM EDTA/PBS and resuspended in 0.05% BSA/0.05% CHAPS-containing HBSS at a density of 0.5×10^7 cells/ml. To 100 μl of this cell suspension was added [^{125}I]-19P2-L31 at a final concentration of 200 pM. In addition, as an NSB (non-specific binding) experiment, 19P2-L31 was added to portions of the cell suspensions at a concentration of 200 nM. The reaction was performed at room temperature for 2.5 hours. After the reaction, B/F separation was carried out with a glass filter GF/F (Wattman) and the radioactivity trapped by the filter was counted with a gamma-counter as a receptor binding amount.

The results of receptor binding experiments using [^{125}I]-19P2-L31 in living cells are shown in Fig. 36.

To 100 μl of a cell suspension, 0.5×10^7 cells/ml, was added [^{125}I]-19P2-L31 at a final concentration of 200 pM, and after a 2.5-hour reaction at room temperature, the amount of [^{125}I]-19P2-L31 bound to the receptor and the non-specific binding amount were determined with a gamma counter. The experiments were performed in triplicate and the mean values and standard deviations were calculated.

In the CHO cells in which hGR3 and UHR-1 were expressed, specific binding of [^{125}I]-19P2-L31 was observed. Those results indicate that the protein

encoded by hGR3 or UHR-1 functions as a specific receptor of 19P2-L31.

[Example 35]

Specific stimulation of arachidonic acid metabolite release from CHO-19P2-9 and CHO-UHR1 by 19P2-L31

The action of 19P2-L31 to stimulate arachidonic acid metabolite release from CHO-19P2-9, CHO-UHR1, and mock CHO was assayed by the same procedure as described in Example 11.

Fig. 37 shows the results of assays of arachidonic acid metabolite releasing activity of 19P2-L31 in CHO-19P2-9 and CHO-UHR1. The experiments were performed in duplicate and the mean results are shown.

In CHO cells with expression of UHR1, too, a comparable degree of arachidonic acid metabolite releasing activity of 19P2-L31 was found as in CHO-19P2-9. Those results indicate that the protein encoded by UHR-1 functions as a specific receptor of 19P2-L31 as does hGR3.

[Example 36]

Assay of the expression of rat tissue ligand polypeptide and rat G protein-coupled receptor (UHR-1) by RT-PCR

(1) Preparation of poly(A)$^+$RNA from rat tissues

Using an 8-week-old rat (♂), poly(A)$^+$RNAs from various tissues were prepared in amounts ranging from about 5 to about 30 μg by the isolation of total RNA with Isogen (Nippon Gene) and subsequent purification with an oligo(dT)cellulose column (Pharmacia).

To completely remove the genome DNA from the poly(A)$^+$RNA fraction, one unit of DNaseI (Gibco BRL, amplification grade) was used to decompose the DNA at room temperature. After addition of 25 mM EDTA, the reaction mixture was incubated at 65℃ for 10 minutes to inactivate the DNaseI. The mixture was diluted to 40 ng/μl with water, and from a 160 ng portion thereof,

a cDNA was synthesized using 10 U AMV reverse transcriptase XL (Takara), 2.5 μM random 9mer (Takara, final concentration 2.5 μM), 10 mM Tris-HCl (pH 8.3), and 0.4 mM each dNTP. The synthetic reaction protocol was 30℃ x 10 minutes followed by 42℃ x 30 min, 99℃ x 5 min, and 5℃ x 5 min. The reaction product was precipitated from ethanol and dissolved in Tricine-EDTA buffer to give a total of 40 μl (4 ng poly(A)⁺RNA/μl).

(2) Construction of a positive control plasmid vector

Rat glyceraldehyde-3-phosphate dehydrogenase (G3PDH, GenBank Accession No. M17701) was amplified by PCR using the cDNA synthesized from the rat pituitary GH3 poly(A)⁺RNA prepared using FastTrack Kit (Invitrogen) in the same manner as in (1) above as a template and Clontech's G3PDH amplification primer set. The UHR-1 was obtained by PCR using the cDNA of GH3, as a template, and the following primers, followed by subcloning into the pCR™ 2.1 Vector of TA Cloning Kit (Invitrogen).

rRECF: 5'-CCTGCTGGCCATTCTCCTGTCTTAC-3' (SEQ ID NO:88)
rRECR: 5'-GGGTCCAGGTCCCGCAGAAGGTTGA-3' (SEQ ID NO:89)

Those were introduced into Escherichia coli JM109 to provide transformants. As the ligand peptide, JM109/pRAV3, already deposited, was used. After each of those transformants was cultured in ampicillin-containing LB medium, the plasmid was purified with Qiagen Plasmid Midi Kit (Qiagen) and, after the concentration was determined from optical density, used as a positive control plasmid vector.

(3) RT-PCR

The cDNA solution and the positive control plasmid vector prepared in (1) and (2) above were used as templates, with or without dilution to a suitable concentration with water. For the amplification of G3PDH, UHR-1, and ligand peptide, Clontech's G3PDH Amplification Primer Set, rRECF/rRECR set, and the

following primer set were used, respectively, at a final concentration of 200 nM.

r19F: 5'-GAAGACGGAGCATGGCCCTGAAGAC-3' (SEQ ID NO:90)

r19R: 5'-GGCAGCTGAGTTGGCCAAGTCCAGT-3' (SEQ ID NO:91)

The reaction mixture consisted of 4 μl of the diluted template, 200 nM each primers, dNTP (final concentration 100 μM each), and KlenTaq (Clontech) as DNA polymerase and used after adjustment to 25 μl with the buffer attached to KlenTaq and water. The amplification reaction conditions were as follows. G3PDH: 94℃ x 1 min. followed by 26 cycles of 98℃ x 10 sec, 65℃ x 20 sec., and 72℃ x 40 sec.; UHR-1 and ligand peptide: 94℃ x 1 min, followed by 34 cycles of 98℃ x 10 sec., 68℃ x 25 sec. The amplification product was electrophoresed on an ethidium bromide-stained 1.2% or 4% agarose gel. The electrophoretogram was photographed by a CCD camera (Fotodyne, Foto/Ecrips) and the concentration of the band was digitalized and quantitated using an analytical software (Advanced American Biotechnology). The data for G3PDH was expressed in pg per 4 ng poly(A)$^+$RNA and the data for UHR-1 and ligand peptide were expressed in pg per 4 ng poly(A)$^+$RNA and, additionally, in the value found by dividing the pg value by pg for G3PDH [Figs. 38 and 39].

As a result, UHR-1 and the ligand peptide were confirmed to be expressed in all tissues. The level of expression of UHR-1 was high in the hypophysis and a broad distribution was found in the brain, too, but the levels of expression in the peripheral tissues were not so high with the exception of the adrenal gland. On the other hand, the level of expression of the ligand peptide was high in the medula oblongata and hypothalamus, among brain tissues, and low in the hypophysis. In the peripheral tissues, the ligand peptide was expressed at comparatively high levels in

the lung, thymus, pancreas, kidney, adrenal, and testis. Those results suggest that UHR-1 and its ligand peptide are playing important roles in various tissues for the modulation of their functions.

[Example 37]

The influence of 19P2-L31 on glucose-induced increase in plasma insulin concentration

Wistar rats (8~10 weeks old, ♂) anesthetized with pentobarbital (65 mg/kg, i.p.) were transitorily dosed with glucose (86 mg/rat) alone or glucose in the same dose plus 19P2-L31 (675 pmol, 2.25 nmol, 6.75 nmol, or 67.5 nmol, per rat) via the common jugular vein, while the blood was serially drawn from the contralateral common jugular vein and the plasma insulin concentration was determined by radioimmunoassay. For this determination, Amersham's insulin assay kit was used.

19P2-L31 in a dose of 675 pmol, 2.25 nmol, or 6.75 nmol, suppressed the first-phase burst of plasma insulin concentration occurring 2 minutes following glucose loading and the second-phase moderate rise in plasma insulin concentration beginning around 6 minutes following administration. Administered in a dose of 67.5 nmol, 19P2-L31 completely inhibited both the first-phase and second-phase increases in insulin concentration [Fig. 40].

[Example 38]

The influence of the ligand polypeptide on the behaviors of mice

The inventors investigated the influence of 19P2-L31 and 19P2-L20 administered into a lateral ventricle of mice on their behaviors. Thus, mature male ICR mice (body weights at operation: ca 35 g) were anesthetized with pentobarbital 50 mg/kg i.p. and immobilized in a rat brain stereotaxic apparatus. The skull was exposed and a hole was drilled with a dental drill for

insertion of a guide cannula into one lateral ventricle. Thus, a stainless steel guide cannula (24G, 5 mm long) for intraventricular medication was inserted with its tip set at AP: +0.6 mm (from bregma), L: 1 mm (left), and H: -1 mm (from dura). The guide cannula was then rigidly secured to the skull with an adhesive. After operation, the mice were reared for at least 3 days for recuperation and then submitted to an experiment for behavioral analysis.

The spontaneous motor activity of mice was measured using a jiggle (spontaneous movement) cage made of clear acrylic resin, 24 x 37 x 30 cm, in a soundproof chamber. The mouse was individually housed in the above cage, and under a 12-hr light-and-dark cycle (ON: 6 to 18 o'clock) and with free access to water and food, the amount of spontaneous motor activity and the amount of rearing were respectively measured. The amount of spontaneous motor activity was measured with Supermex (Muromachi Machinery). The peptide or phosphate buffered saline (PBS) was administered at 2:30 ± 30 min., p.m. For administration, a stainless steel microinjection cannula (30 G, 6 mm long) was passed through the guide cannula. The microinjection cannula was connected to a microsyringe pump via a Teflon tube and either PBS or a PBS solution of the peptide was infused at a flow rate of 2 μl/min for 2 minutes. The microinjection cannula was left inserted for at least 2 minutes after completion of infusion and, then, removed and the amount of spontaneous motor activity was measured.

The results were expressed in mean ± S.E.M. and the significance of the relative effect of the peptide and PBS treatments on motor activity was analyzed by Student's t-test. The difference at the 5% level of significance ($p<5\%$) on a two-tailed basis was regarded as being statistically significant. It is clear from

Fig. 41 that when 10 nmol of 19P2-L31 was administered, the spontaneous motor activity of mice was increased significantly during the period from 70 to 105 minutes after administration. The rearing behavior also showed a significant change in the like fashion. When, 1 nmol of 19P2-L31 was administered, no change was found in spontaneous activity and the amount of rearing was decreased significantly only at 105 minutes following administration [Fig. 42]. With 0.1 nmol of 19P2-31, the amount of spontaneous motor activity was increased significantly at 25, 40, and 70 minutes following administration. The amount of rearing also showed a similar trend but did not change significantly [Fig. 43]. With 0.01 nmol of 19P2-L31, spontaneous motor activity was increased significantly at 20 and 40 minutes following administration. The amount of rearing also showed a similar tendency toward increase but the change was not significant [Fig. 44].

[Example 39]

The influence of the ligand peptide on reserpine-induced hypothermia in mice

Mature male ICR mice (body weights at operation: ca 35 g) were anesthetized with pentobarbital 50 mg/kg i.p. and immobilized in a rat brain stereotaxic apparatus. The skull was exposed and a hole was drilled with a dental drill for indwelling a guide cannula in one lateral ventricle. A stainless steel guide cannula for intraventricular medication (24 G, 5 mm long) was inserted with its tip set at AP: +0.6 mm (from bregma), L: 1 mm (left), H: -1 mm (from dura). The guide cannula was rigidly secured to the skull with an adhesive. After operation, the mice were reared for at least 3 days for recuperation and the body temperature was then measured. Then, reserpine (Apopron Inj. 1 mg, Daiichi Pharmaceutical), 3 mg/kg, was injected subcutaneously, and 15 hours later the

mice were transferred to individual cages for body temperature measurement. A stainless steel microinjection cannula (30 G, 6 mm long) was passed into the guide cannula. The microinjection cannula was connected to a microinjection syringe pump via a Teflon tube and PBS or a PBS solution of the peptide was infused at a flow rate of 2 μl/min. for 2 minutes. The microinjection cannula was left installed for at least 2 minutes following completion of infusion and, then, removed and the rectal temperature was measured.

The results were expressed in mean ± S.E.M. and the significance of the relative effect of the peptide and PBS treatments on body temperature was analyzed by Student's t-test. The difference at the 5% level of significance on a two-tailed basis was regarded as being statistically significant. It is clear from Fig. 45 that when 10 nmol of 19P2-L31 was administered, the body temperature depressed by reserpine was elevated significantly as compared with the PBS control group. This elevation of body temperature peaked at 45 minutes following administration of 19P2-L31. On the other hand, no difference was found between the 19P2-L20 1 nmol group and the control group.

[Example 40]

The influence of the ligand polypeptide on rat blood pressure

The inventors of the present invention studied the influence of 19P2-L31 injected into the area postrema (AP) of medula oblongata on rat blood pressure. Mature male Wistar rats (body weights at operation: ca 300 g) were anesthetized with pentobarbital 50 mg/kg i.p. and immobilized in a rat brain stereotaxic apparatus. The incisal bar was set 3.3 mm below the interoral line. The skull was exposed and a hole was drilled with a dental drill for indwelling a guide cannula. In addition, anchor screws were embedded in 2

positions around the hole. A stainless steel guide cannula, AG-12 (inside dia. 0.4 mm, out. dia. 0.5 mm, Acom), was inserted with its tip situated in the superior domain of the area postrema. For this purpose, the guide cannula was inserted from the anterior direction at an angle of 20 degrees with the vertical direction (Fig. 46; the figure shows a microinjection cannula which is longer than the guide cannula by 1.0 mm). The stereotaxic coordinates of AP: -0.6 mm (from interoral line), L: 0.0 mm, H: +1.5 mm (from interoral line) were used with reference to the atlas of Paxinos and Watson (1986). The guide cannula was secured to the skull with an instant adhesive, a dental cement, and said anchor screws. In the guide cannula, a stainless steel dummy cannula, AD-12 (out. dia. 0.35 mm, Acom), was inserted and secured in position with a cap nut (Acom). Thereafter, the rats were reared in individual cages.

The animals were reared for about a week following cannulation for recuperation and a surgery was performed for measurement of conscious blood pressure. Thus, the rat was anesthetized with pentobarbital 50 mg/kg i.p. and immobilized in supine position on a dissection pad, and the left femoral artery was exposed. A polyethylene tube, SP35 (in. dia. 0.5 mm, out. dia. 0.9 mm, Natsume Seisakusho), was cut to about 60 cm in length and the cut tube was filled with 200 U/ml heparin-containing saline and inserted into the femoral artery over a distance of about 2.5 cm and secured in position. The other end of the tube was passed beneath the dorsal skin and exposed from the cervical (dorsal) region.

After one night following operation, the polyethylene tube was connected to a pressure transducer (Spectramed) and the blood pressure was measured. After the blood pressure reading had become steady, the

cap nut and dummy cannula were removed from the rat skull and, instead, a stainless steel microinjection cannula, AMI13 (in. dia. 0.17 mm, out. dia. 0.35 mm, Acom), connected to a Teflon tube (50 cm long, 0.1 mm in. dia., 0.4 mm out. dia., Acom), was inserted. The length of the microinjection cannula was adjusted beforehand so that its tip would be exposed from the guide cannula over a distance of 1 mm [Fig. 46]. The other end of the Teflon tube was connected to a microsyringe pump and 2 μl of either PBS or a PBS solution of 19P2-L31 was injected into the area postrema at a flow rate of 1.0 μl/min.

After blood pressure measurement, the microinjection cannula used for injection of 19P-L31 was removed and, instead, a microinjection cannula for infusion of a dye (Evans blue) was installed. The dye was similarly infused at a flow rate of 1.0 μl/min for 2 minutes and after a waiting time of about 3 minutes the microinjection cannula was removed. The rat was decapitated and the brain was quickly enucleated and frozen. Using a cryostat, frozen sections were prepared and the infusion position of the dye was confirmed.

The above experiment revealed that the infusion of 10 nmol of 19P2-L31 into the area postrema caused a rise in blood pressure. A typical example of pulse wave and mean blood pressure is shown in Fig. 47.

[Example 41]

The influence of the ligand polypeptide on the plasma pituitary hormone level

The inventors of the present invention studied the influence of 19P2-L31 injected into the third ventricle on the plasma pituitary hormone levels. Mature male Wistar rats (body weights at operation: ca 290-350 g) were anesthetized with pentobarbital 50 mg/kg i.p. and each animal was immobilized in a rat

brain stereotaxic apparatus. The incisal bar was set 3.3 mm below the interoral line. The skull was exposed and using a dental bar a hole was drilled for indwelling a guide cannula. In addition, an anchor screw was embedded in one position around the hole. A stainless steel guide cannula, AG-12 (in. dia. 0.4 mm, out. dia. 0.5 mm, Acom), was inserted with its tip positioned in the superior domain of the third ventricle. The stereotaxic coordinates of AP: +7.2 mm (from interoral line), L: 0.0 mm, H: +2.0 mm (from interoral line) were used with reference to the atlas of Paxinos and Watson (1986). The guide cannula was rigidly secured to the skull with an instant adhesive, a dental cement, and said anchor screw. In the guide cannula, a stainless steel dummy cannula, AD-12 (out. dia. 0.35 mm, Acom) was passed and secured in position with a cap nut (Acom). After operation, the rats were reared in individual cages for at least 3 days for recuperation and then submitted to the experiment.

The rat operated on as above was anesthetized with pentobarbital 50 mg/kg i.p. and immobilized in supine position on a dissection pad. After the bilateral jugular veins were exposed, 400 μl of blood was collected into a 1 ml tuberculin syringe with a 24 G needle (both from Terumo). To prevent clotting, the syringe was filled with 20 μl of 200 U/ml heparin-containing saline ahead of time. The cap nut and dummy cannula were removed from the rat skull and, instead, a stainless steel microinjection cannula, AMI13 (in. dia. 0.17 mm, out. dia. 0.35 mm, Acom), connected to a Teflon tube (50 cm long, 0.1 mm in. dia, 0.4 mm out. dia. Acom) was inserted. The length of the microinjection cannula was adjusted beforehand so that its tip would be exposed from the guide cannula over a distance of 1 mm. The other end of the Teflon tube was connected to a microsyringe pump and 10 μl of PBS or a

PBS solution of 19P2-L31 was injected into the third ventricle at a flow rate of 2.5 μl/min. After a waiting time of 1 minute following completion of injection, the microinjection cannula was removed and the dummy cannula was reinstalled and secured with the cap nut. Immediately before intraventricular administration and 10, 20, 30, 40, and 60 minutes after the start of intraventricular administration, 400 μl of blood was collected from the jugular vein. Each blood sample was centrifuged (5,000 rpm, 10 min.) using a high-speed refrigerated microcentrifuge (MR-150, Tomy precision Industry) and the supernatant (plasma) was recovered. The pituitary hormones [prolactin, luteinizing hormone (LH), adrenocorticotropic hormone (ACTH), and thyrotropin (TSH), and growth hormone (GH)] in the plasma were respectively assayed by radioimmunoassays.

The results were expressed in mean ± S.E.M. For the significance testing of the difference between the 19P2-L31/PBS group and the PBS group, Student's t-test was used. As a test for statistical significance, the 5% level was used. It can be seen from Fig. 48 that the plasmal level of growth hormone in the 19P2-L31 group was significantly decreased at 20 minutes after injection of 50 nmol into the third ventricle. The trend toward decrease was also observed at 10, 30, and 40 minutes as well but the changes were not significant. At 60 minutes after injection, there was no difference from the control group. The plasma prolactin, LH, ACTH, and TSH levels were not altered significantly

[Example 42]

Effects of ligand polypeptide on plasma growth hormone (GH) level in freely moving rats

Mature male Wistar rats were anesthetized with pentobarbital 50 mg/kg i.p. and, as in Example 41, a stainless-steel guide cannula AG-12 (0.4 mm in. dia.,

0.5 mm out. dia., EICOM) was implanted in position with its tip situated in the upper part of the third ventricle. After the operation the rats were housed in individual cages and kept for at least 3 days for recuperation and, then, a cannula (30 cm long, 0.5 mm in. dia., 0.9 mm out. dia., Natsume Seisakusho) filled in with heparin (200 U/ml)-containing saline was inserted into the right atrium from the right jugular vein under pentobarbital anesthesia. The rats were maintained overnight for complete arousal from anesthesia and then transferred to transparent acrylic cages (30 cm x 30 cm x 35 cm). A 1 ml tuberculin syringe with a 24-G needle (both by Termo) was connected to the cannula inserted in the atrium and 300μl of blood was drawn. To prevent clotting, the syringe was filled in with 20μl of saline containing 200 U/ml of heparin beforehand. A stainless-steel microinjection cannula (0.17 mm in. dia., 0.35 mm out. dia., EICOM) connected to Teflon tube (50 cm long, 0.1 mm in. dia., 0.4 mm out. dia., EICOM) was inserted into the guide cannula positioned in the third ventricle. The length of the microinjection cannula was adjusted beforehand so that its tip would be extend 1 mm from the guide cannula. One end of the Teflon tube was connected to a microsyringe pump and either PBS or 19P2-L31 dissolved in PBS was injected, in a total volume of 10μl, into the third ventricle at a flow rate of 2.5μl/min. Ten minutes after initiation of administration into the third ventricle, 5μg/kg GHRH-saline was administered via the cannula inserted into the atrium. Immediately before initiation of intraventricular administration and 10, 20, 30, 40, and 60 minutes after administration of GHRH, 300μl portions of blood were drawn from the jugular vein. Each blood sample was centrifuged (5,000 rpm, 10 min.) and the supernatant (plasma) was

recovered. The concentrations of GH in the plasma were determined by radioimmunoassay.

The results were expressed as a mean ±S.E.M. To test for significant difference between the group treated with 19P2-L31 dissolved in PBS and the control group treated with PBS alone, Student's t-test was used. According to the two tailed test, $p<0.05$ was assumed to be the minimal level of significance. As shown in Fig. 49, administration of 5μg/kg of GHRH elevated the plasma GH level. However, when 50 nmol of 19P2-L31 was administered into the third ventricle, the GHRH-induced elevation of plasma GH was significantly inhibited.

[Example 43]

Preparation of rabbit anti-bovine 19P2-L31 antibodies

Synthetic peptides containing partial 19P2-L31 sequence [peptide-I: SRAHQHSMEIRTPDC (SEQ ID NO:92), peptide-II: CAWYAGRGIRPVGRFNH2 (SEQ ID NO:93), and peptide-III: CEIRTPDINPAWYAG (SEQ ID NO:94) were conjugated with KLH according to the standard method. Each peptide conjugate (600μg as a peptide) dissolved in saline was mixed with Freund's complete adjuvant, and the resultant emulsion was subcutaneously injected into three rabbits (NZW, male, 2.5 kg) respectively. Hyperimmunization was carried out three times in total at the same dose of the conjugate as the first injection with Freund's imcomplete adjuvant every three weeks. Antibody titers were determined as follows. Two weeks after the last immunization, blood samples were obtained from the vein of the immunized rabbits respectively. After being incubated at 37℃ for 1 hour, the blood samples were kept at 4℃ over night. Sera were then prepared by means of centrifugation. An aliquot (100μl) of each serum sample diluted properly was introduced into 96-well polystyrene microplates

which were pre-coated with goat anti-rabbit IgG (Fc) antibodies, and then the microplates were incubated at 4℃ for 16 hours. After removing the sera, horse radish peroxidase (HRP)-conjugated peptide-I, II, and III were added to the wells respectively, and then the microplates were incubated at room temperature for 4 hours. After removing the peptides, coloring reaction was done by adding a substrate. The reaction was stopped by adding 100μl of a stopping solution, and then the absorbance at 450 nm in each well was measured. As shown in Fig. 50, serum samples obtained from the rabbits after the immunization showed binding activities to HRP-conjugated peptides respectively. However, none of binding activities was detected in sera prepared before the immunization. These results indicated that the rabbits received the immunization produced antibodies against peptide-I, II, and III, respectively. To prepare purified IgG antibody fractions, sera obtained from the immunized rabbits was percipitated with anmonium sulfate. The resultant precipitates were dissolved in borate buffer, and then dialyzed with the same buffer. The IgG fractions thus obtained were then subjected onto affinity columns conjugated with peptide-I or 19P2-L31 respectively. After washing the columns with borate buffer and following with acetate buffer (100 mM, pH 4.5), antibodies bound to the column were eluted with glycine buffer (200 mM, pH 2.0). After being neutralized with 1M Tris, the eluents were used as purified antibodies respectively.

[Example 44]

Inhibitory activity of antibodies against the release of arachidonic acid metabolites induced by 19P2-L31

The purified antibodies prepared as described in Example 43 were tested their inhibitory activity

against the release of arachidonic acid metabolites induced by 19P2-L31. The antibodies diluted as indicated in Fig. 51 were mixed with 19P2-L31 (5×10^{-10}M) at room temperature for 1 hour, and then the release of arachidonic acid metabolites was examined as described in Example 11. As shown in Fig. 51, the highest inhibitory activity was observed in anti-peptide-II antibodies.

[Example 45]

Based on the DNA sequence coding the murine-derived ligand polypeptide (Figure 32) obtained in Example 29, two primers, were synthesized.

rFBG:5'-AGATTGGCATCATCCAGGAAGACGGAGCAT-3'(SEQ ID NO:95)

rRSA:5'-GTCGACTCAGCAGCACTGTCTTCTCGAGCTG-3'

(SEQ ID NO:96)

Using the cDNA prepared using 0.5 ng of m01.2212.....121urine genomic DNA (Mouse BALB/c genomic DNA as a template and PCR was carried out.

50 μl of reaction mixture comprises 200nM each of synthetic DNA primer, o.5 nM of template DNA, 0.25mM of dNTPs, 0.5 μl of E × Taq polymerase, and buffers attached with enzyme. An amplification reaction was carried out in 30 cycles of 95℃ x 30 sec and 67℃ x 60 sec. The amplification product was identified by 1.2% agarose gel electrophoresis with ethidium bromide staining and a 1 kb (approx.) band was recovered and subcloned using TA Cloning Kit (Invitrogen). This ligation mixture was used to transform E. coli JM109 and clones harboring the inserted fragment were selected on ampicillin- and X-gal-containing LB agar. A white clone was isolated to provide a transformant, Escherichia coli JM109/pmGB3. This clone was cultured overnight in an ampicillin-containing LB medium and, using an automatic plasmid extractor, a plasmid DNA was

prepared. A portion of the DNA thus prepared was subjected to a sequencing reaction using ABI Dye Terminator Cycle Sequencing Kit (ABI) and analyzed with a fluorescent automated sequencer. The oligonucleotide sequence data thus obtained was analyzed with DNASIS (Hitachi System Engineering) (Fig. 53). The underscored sequences correspond to the primer sequences.

The nucleotide sequence determined in this manner was compared with the sequence of SEQ ID NO:2, 46, or 60. As a result, the DNA fragment inserted in the plasmid pmGB3 horbored by Escherichia coli JM109/pmGB3 was found to code for a novel mouse ligard polypeptide [Fig. 54].

[Example 46]

The influence of 19P2-L31 on prolactin secretion from pituitary cell line RC-4B/C

The rat pituitary cell line RC-4B/C (Hurbain-Kosmath et al., In Vitro Cell. Dev. Biol., 26, 431-440 (1990)) was seeded on a 12-well plate (Sumitomo Bakelite) at a density of $1x10^5$ cells/well and cultured for 2 days. The medium composition was as suggested in the above literature (DMEM (Nissui): α-MEM (Gibco) = 1:1, 10% fetal calf serum, 1.5 g/l glucose (Wako), 0.2 mg/ml BSA (Sigma), 0.5% nonessential amino acids solution (Flow Laboratories), 15 mM HEPES (Wako) pH 7.3, 2.5 ng/ml EGF (Genzyme), 50 ng/ml gentamicin (Gibco)) and the cultivation was carried out under 10% CO_2 at 34℃.

The cultured cells were washed with 3 portions of incubation buffer (DMEM: α-MEM = 1:1, 0.5 g/l glucose, 0.1% BSA, 0.5% nonessential amino acids solution, 15 mM HEPES pH 7.3) and after addition of the same buffer, a preincubation was carried out under 10% CO_2 at 34℃ for 15 minutes. The cells were re-washed with two portions of the same buffer. Then, a preparation of bovine

19P2-L31 peptide (SEQ ID NO:5) in incubation buffer was added at the varying concentration shown in Fig. 55 and an incubation was performed under 10% CO_2 at 34℃ for 30 minutes. To remove the floating cells, the culture was centrifuged with a high-speed microcentrifuge and the supernatant was stored at -30℃.

The amount of prolactin in the culture supernatant sample obtained by the above procedure was determined with Rat Prolactin [^{125}I] Assay System (Amersham).

It can be seen from Fig. 55 that addition of 19P2-L31 caused a concentration-dependent increase in prolactin secretion from RC-4B/C cells. The mark ** in the diagram indicates a significance with not less than 99% confidence versus the experiment without addition of 19P2-L31 as analyzed by Student's t-test.

[Example 47]

The influence of 19P2-L31 on prolactin secretion from primary cultured rat pituitary cells

The primary cultured rat pituitary cells were prepared according to the method of Shiota et al. (Acta Endocrinologica, 106, 71-78 (1984).

A female Fischer 344/N rat (SLC) at about 11 lactation days was decapitated to death and the anterior lobe of hypophysis was isolated. The isolated pituitary specimen was washed with buffer A [137 mM NaCl (Wako), 5 mM KCl (Wako), 0.7 mM Na_2HPO_4 (Wako), 50 μg/ml gentamicin (Gibco)] and treated with enzyme solution I [0.4% collagenase A (Boehringer-Mannheim), 10 μg/ml DNase (Sigma), 0.4% BSA (Sigma), 0.2% glucose (Wako)] in buffer A at 37℃ for 1 hour. After the pituitary preparation was dispersed into cells by pipetting, the dispersion was centrifuged to remove the supernatant and the pellet was suspended in enzyme solution II (0.25% pancreatin (Sigma) in buffer A and incubated at 37℃ for 8 minutes. The reaction was

stopped by adding fetal calf serum and the reaction mixture was centrifuged to remove the supernatant. The resulting cells were suspended in DMEM-I (DMEM: Dulbecco's minimum essential medium, 10% fetal calf serum, 20 mM HEPES pH 7.3, 50 U/ml penicillin, 50 μ g/ml streptomycin), passed through a cell strainer (Falcon) to remove cell conglomerates and fibrous contaminants, and washed with 2 portions of DMEM-I. The cells thus obtained were diluted in DMEM-I, seeded at a cell density of 1.5×10^5/well, and cultured under 5% CO_2 at 37℃ for 4 days.

On day 3 of culture the medium was replaced with fresh one and on day 4 a sample of culture supernatant was prepared. Thus, cells were washed with 3 portions of DMEM-II (DMEM, 0.2% BSA, 20 mM HEPES pH 7.3), DMEM-II was added, and the mixture was preincubated under 5% CO_2 at 37℃ for 1 hour. After washing with 2 portions of DMEM-II, a solution of 19P2-L31 peptide (amide form of SEQ ID NO:5) in DMEM-II was added at the varying concentration shown in Fig. 56 and the reaction was carried out under 5% CO_2 at 37℃ for 1 hour. The supernatant was recovered, centrifuged to remove floating cells, and stored at -30℃ for use as a supernatant sample.

The concentration of prolactin in the culture supernatant was determined with Rat Prolactin [^{125}I] Assay System (Amersham).

It can be seen from Fig. 56 that addition of 19P2-L31 caused a concentration-dependent increase in prolactin secretion from the primary cultured pituitary cells. The mark ** in the diagram indicates that as analyzed by Student's t-test the particular value is statistically significant at $p<0.01$ compared with the corresponding value found without addition of 19P2-L31. The mark * indicates that as analyzed by Student's t-test the particular value is significant at $p<0.05$

compared with the corresponding value found without addition of 19P2-L31.

[Example 48]

The time course of expression of UHR-1 gene in the rat placenta

From female rats at 12 weeks of age, placental samples were isolated on days 11, 14, 17, and 20 of gestation. Those tissues were quickly frozen in liquid nitrogen and stored at -80℃. For the preparation of mRNA, each frozen tissue was homogenized with Isogen solution (Nippon Gene) and then total RNA was prepared in accordance with its manual. From 1 mg of each total RNA, mRNA was prepared using a mRNA Purification Kit (Pharmacia). After 1 μg of the mRNA was treated with DNase I (Amprification Grade, Gibco BRL), 160 ng was taken and synthesized a cDNA using a RNA PCR Kit (Takara Shuzo) with random 9mer primers at 42℃ for 30 minutes. Each of the cDNAs thus prepared was dissolved in 40 μl of TE buffer. Assay of the amount of expression of UHR-1 gene was carried out using ABI PRISM 7700 Sequence Detector (Perkin-Elmer). For the reaction, rU1F (5'-AACCCCTTCATCTATGCGTGG-3') and rU1R (5'-ATATTCTGGCCATGAGGCAC-3' (SEQ ID NO:98)) were used as primers and rU1P (5'-TTCCGAGAGGAGCTACGCAAGATGCTTC-3'(SEQ ID NO:99)) as the fluorescence-labeled probe. The reaction mixture was prepared using the proprietary reagent kit TaqMan PCR Core Reagent Kit (Perkin-Elmer) in accordance with the manual. In this procedures, 4 μl of a 40-fold dilution of the sample cDNA in TE buffer was added to the reaction mixture. A DNA fragment for which the number of moles of UHR-1 gene was determined by measuring the absorbance at 260 nm was diluted, and then used as templates for PCR to obtain a calibration curve for quantification. PCR was performed under the conditions of 50℃ x 2 min. and

95℃ x 10 min, followed by 40 cycles of 95℃ x 10 sec. and 55℃ x 1.5 min. The results indicated that the amount of expression of UHR-1 gene in the rat placenta increased remarkably with an increasing gestation period.

[Example 49]

The influence of 19P2-L31 on plasma prolactin concentration in rats

(1) Activity of 19P2-L31 on male rats

The inventors studied the influence of 19P2-L31 administered i.v. on plasma prolactin concentration on male rats. Mature male Fischer rats (body weights: ca 150~180 g) were anesthetized with urethane 1.5 mg/kg i.p. and each sides of the right jugular vein were exposed by operation, 20 minutes after anesthesia. 15 minutes after the operation, a solution of 19P2-L31 (50 or 500 nmol/kg) in 1% bovine serum albumin (BSA)-saline or, in the control group, 1% BSA-saline was administered by using a 1 ml tuberculin syringe. Immediately before initiation of intravenous administration and 2, 5, 10, and 20 minutes after administration, 200 μl of blood was serially drawn from the jugular vein. To prevent clotting, the syringe was filled in with 10 μl of 150 U/ml heparin-saline ahead of time. Each blood sample was centrifuged (10,000 rpm, 15 min.) using a high-speed refrigerated microcentrifuge (MR-150, Tomy Precision Machinery) and the supernatant (plasma) was recovered. The amount of prolactin contained in the plasma was determined with a radioimmunoassay kit (Amersham). The time course of plasma prolactin concentration was expressed in mean ± S.E.M. and the significance of difference between the 19P2-L31/1% BSA-saline group and 1% BSA-saline group was tested by Dunnett's method. 5% level of significance ($p \leqq 0.05$) was used. It is ır from Fig. 58 that administration of 19P2-L31 in a

dose of 500 nmol/kg caused a significant increase in plasma prolactin concentration, compared with the control group, at 2 minutes following administration.

(2) Activity of 19P2-L31 on female rats

Subsequently, the inventors studied the influence of 19P2-L31 administered i.v. on plasma concentration on female rats. Sexual cycles of mature female Fischer rats (body weight : ca 140 to 160g) were determined by ostium vaginae test, and the influence of 19P2-L31 administered i.v. on plasma prolactin concentration was studied by the same method as described on Example 49-(1) mentioned above. The time course of plasma prolactin concentration was expressed in mean ± S.E.M. and the significance of difference between the 19P2-L31/1% BSA-saline group and the 1% BSA-saline group was tested by Dunnett's method. The 5% level of significance ($p \leqq 0.05$) was used. It is clear from Fig. 59 that administration of 19P2-L31 in a dose of 50 nmol/kg caused a significant increase in plasma prolactin concentration, compared with the control group, at 5 minutes following administration. It is also clear from Fig. 59 that administration of 19P2-L31 on female rats in a dose of about 1/10 showed the equevalent or superior activities compared with the case of the administration on male rats. In addition, As shown in Fig. 60, when the time course of plasma prolactin concentration was determined among the sexual cycle, a significant increase in plasma prolactin concentration was observed in estrus. This indicates that the effect of 19P2-L31 is different depending on the sexual cycles of the female rats.

[Preparation Example 1]

Fifty milligrams of the compound as obtained in Example 21 is dissolved in 50 ml of distilled water for injection (Japanese pharmacopoeial), and distilled water for injection (Japanese pharmacopoeial) is added

thereto to make 100 ml. The resulting solution is filtered under a germ-free condition, and each vial for injection is filled in with 1 ml of the filtrate, freeze-dried and sealed therein also under a germ-free condition.

[Preparation Example 2]

One hundred milligrams of the compound as obtained in Example 21 is dissolved in 50 ml of distilled water for injection (Japanese pharmacopoeial), and distilled water for injection (Japanese pharmacopoeial) is added thereto to make 100 ml. The resulting solution is filtered under a germ-free condition, and each vial for injection is filled in with 1 ml of the filtrate, freeze-dried and sealed therein also under a germ-free condition.

[Sequence Listing]

(1) GENERAL INFORMATION:

(i) APPLICANT:

(A) NAME: Takeda Chemical Industries, Ltd.

(B) STREET: 1-1, Doshomachi 4-chome, Chuo-ku

(C) CITY: Osaka

(D) STATE: Osaka

(E) COUNTRY: Japan

(F) POSTAL CODE (ZIP): 541

(ii) TITLE OF INVENTION: Polypeptides, Their Production and Use

(iii) NUMBER OF SEQUENCES: 94

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE:

(B) COMPUTER:

(C) OPERATING SYSTEM:

(D) SOFTWARE:

(v) CURRENT APPLICATION DATA:

APPLICATION NUMBER:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 98

(B) TYPE: Amino acid

(C) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Met Lys Ala Val Gly Ala Trp Leu Leu Cys Leu Leu Leu Leu Gly Leu
1 5 10 15
Ala Leu Gln Gly Ala Ala Ser Arg Ala His Gln His Ser Met Glu Ile
20 25 30
Arg Thr Pro Asp Ile Asn Pro Ala Trp Tyr Ala Gly Arg Gly Ile Arg
35 40 45
Pro Val Gly Arg Phe Gly Arg Arg Arg Ala Ala Pro Gly Asp Gly Pro
50 55 60
Arg Pro Gly Pro Arg Arg Val Pro Ala Cys Phe Arg Leu Glu Gly Gly
65 70 75 80
Ala Glu Pro Ser Arg Ala Leu Pro Gly Arg Leu Thr Ala Gln Leu Val
85 90 95
Gln Glu

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 294

(B) TYPE: Nucleic acid

(C) STRANDENESS: Double

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA

(xi) FEATURE

(C) IDENTIFICATION METHOD: S

(x) SEQUENCE DESCROPTION; SEQ ID NO:2:

ATGAAGGCGG TGGGGGCCTG GCTCCTCTGC CTGCTGCTGC TGGGCCTGGC CCTGCAGGGG 60
GCTGCCAGCA GAGCCCACCA GCACTCCATG GAGATCCGCA CCCCCGACAT CAACCCTGCC 120
TGGTACGCRG GCCGTGGGAT CCGGCCCGTG GGCCGCTTCG GCCGGCGAAG AGCTGCCCYG 180

GGGGACGGAC CCAGGCCTGG CCCCCGGCGT GTGCCGGCCT GCTTCCGCCT GGAAGGCGGY 240
GCTGAGCCCT CCCGAGCCCT CCCGGGGCGG CTGACGGCCC AGCTGGTCCA GGAA 294

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29

(B) TYPE: Amino acid

(C) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Ser Arg Ala His Gln His Ser Met Glu Ile Arg Thr Pro Asp Ile Asn
1 5 10 15
Pro Ala Trp Tyr Ala Gly Arg Gly Ile Arg Pro Val Gly
20 25

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19

(B) TYPE: Amino acid

(C) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Thr Pro Asp Ile Asn Pro Ala Trp Tyr Ala Gly Arg Gly Ile Arg Pro
1 5 10 15
Val Gly Arg
19

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 31

(B) TYPE: Amino acid

(C) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Ser Arg Ala His Gln His Ser Met Glu Ile Arg Thr Pro Asp Ile Asn
1 5 10 15
Pro Ala Trp Tyr Ala Gly Arg Gly Ile Arg Pro Val Gly Arg Phe
20 25 30

(2) INFORMATION FOR SEQ ID NO:6:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32
(B) TYPE: Amino acid
(C) TOPOLOGY: Linear
(ii) MOLECULE TYPE: Peptide
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Ser Arg Ala His Gln His Ser Met Glu Ile Arg Thr Pro Asp Ile Asn
1 5 10 15
Pro Ala Trp Tyr Ala Gly Arg Gly Ile Arg Pro Val Gly Arg Phe Gly
20 25 30

(2) INFORMATION FOR SEQ ID NO:7:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33
(B) TYPE: Amino acid
(C) TOPOLOGY: Linear
(ii) MOLECULE TYPE: Peptide
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Ser Arg Ala His Gln His Ser Met Glu Ile Arg Thr Pro Asp Ile Asn
1 5 10 15
Pro Ala Trp Tyr Ala Gly Arg Gly Ile Arg Pro Val Gly Arg Phe Gly
20 25 30
Arg
33

(2) INFORMATION FOR SEQ ID NO:8:
(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20
(B) TYPE: Amino acid
(C) TOPOLOGY: Linear
(ii) MOLECULE TYPE: Peptide
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Thr Pro Asp Ile Asn Pro Ala Trp Tyr Ala Gly Arg Gly Ile Arg Pro
1 5 10 15
Val Gly Arg Phe
20

(2) INFORMATION FOR SEQ ID NO:9:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21
(B) TYPE: Amino acid
(C) TOPOLOGY: Linear
(ii) MOLECULE TYPE: Peptide
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Thr Pro Asp Ile Asn Pro Ala Trp Tyr Ala Gly Arg Gly Ile Arg Pro
1 5 10 15
Val Gly Arg Phe Gly
20

(2) INFORMATION FOR SEQ ID NO:10:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22
(B) TYPE: Amino acid
(C) TOPOLOGY: Linear
(ii) MOLECULE TYPE: Peptide
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Thr Pro Asp Ile Asn Pro Ala Trp Tyr Ala Gly Arg Gly Ile Arg Pro
1 5 10 15
Val Gly Arg Phe Gly Arg
20

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 87

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Double

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

AGCAGAGCCC ACCAGCACTC CATGGAGATC CGCACCCCCG ACATCAACCC TGCCTGGTAC 60

GCRGGCCGTG GGATCCGGCC CGTGGGC 87

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 57

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Double

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

ACCCCCGACA TCAACCCTGC CTGGTACGCR GGCCGTGGGA TCCGGCCCGT GGGCCGC 57

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 93

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Double

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

AGCAGAGCCC ACCAGCACTC CATGGAGATC CGCACCCCCG ACATCAACCC TGCCTGGTAC 60

GCRGGCCGTG GGATCCGGCC CGTGGGCCGC TTC 93

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 96

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Double

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

AGCAGAGCCC ACCAGCACTC CATGGAGATC CGCACCCCCG ACATCAACCC TGCCTGGTAC 60

GCRGGCCGTG GGATCCGGCC CGTGGGCCGC TTCGGC 96

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 99

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Double

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

AGCAGAGCCC ACCAGCACTC CATGGAGATC CGCACCCCCG ACATCAACCC TGCCTGGTAC 60

GCRGGCCGTG GGATCCGGCC CGTGGGCCGC TTCGGCCGG 99

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 60

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Double

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

ACCCCCGACA TCAACCCTGC CTGGTACGCR GGCCGTGGGA TCCGGCCCGT GGGCCGCTTC 60

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 63
(B) TYPE: Nucleic acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: cDNA
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

ACCCCCGACA TCAACCCTGC CTGGTACGCR GGCCGTGGGA TCCGGCCCGT GGGCCGCTTC 60
GGC 63

(2) INFORMATION FOR SEQ ID NO:18:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 66
(B) TYPE: Nucleic acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: cDNA
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

ACCCCCGACA TCAACCCTGC CTGGTACGCR GGCCGTGGGA TCCGGCCCGT GGGCCGCTTC 60
GGCCGG 66

(2) INFORMATION FOR SEQ ID NO:19:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 91
(B) TYPE: Amino acid
(C) TOPOLOGY: Linear
(ii) MOLECULE TYPE: Peptide
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Leu Val Leu Val Ile Ala Arg Val Arg Arg Leu His Asn Val Thr Asn
1 5 10 15
Phe Leu Ile Gly Asn Leu Ala Leu Ser Asp Val Leu Met Cys Thr Ala
20 25 30
Cys Val Pro Leu Thr Leu Ala Tyr Ala Phe Glu Pro Arg Gly Trp Val

35 40 45

Phe Gly Gly Gly Leu Cys His Leu Val Phe Phe Leu Gln Pro Val Thr
50 55 60

Val Tyr Val Ser Val Phe Thr Leu Thr Thr Ile Ala Val Asp Arg Tyr
65 70 75 80

Val Val Leu Val His Pro Leu Arg Arg Arg Ile
85 90

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 59

(B) TYPE: Amino acid

(C) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Gly Leu Leu Leu Val Thr Tyr Leu Leu Pro Leu Leu Val Ile Leu Leu
1 5 10 15

Ser Tyr Val Arg Val Ser Val Lys Leu Arg Asn Arg Val Val Pro Gly
20 25 30

Cys Val Thr Gln Ser Gln Ala Asp Trp Asp Arg Ala Arg Arg Arg Arg
35 40 45

Thr Phe Cys Leu Leu Val Val Val Val Val Val
50 55

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 370

(B) TYPE: Amino acid

(C) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Met Ala Ser Ser Thr Thr Arg Gly Pro Arg Val Ser Asp Leu Phe Ser
1 5 10 15

Gly Leu Pro Pro Ala Val Thr Thr Pro Ala Asn Gln Ser Ala Glu Ala

20 25 30

Ser Ala Gly Asn Gly Ser Val Ala Gly Ala Asp Ala Pro Ala Val Thr

35 40 45

Pro Phe Gln Ser Leu Gln Leu Val His Gln Leu Lys Gly Leu Ile Val

50 55 60

Leu Leu Tyr Ser Val Val Val Val Val Gly Leu Val Gly Asn Cys Leu

65 70 75 80

Leu Val Leu Val Ile Ala Arg Val Arg Arg Leu His Asn Val Thr Asn

85 90 95

Phe Leu Ile Gly Asn Leu Ala Leu Ser Asp Val Leu Met Cys Thr Ala

100 105 110

Cys Val Pro Leu Thr Leu Ala Tyr Ala Phe Glu Pro Arg Gly Trp Val

115 120 125

Phe Gly Gly Gly Leu Cys His Leu Val Phe Phe Leu Gln Pro Val Thr

130 135 140

Val Tyr Val Ser Val Phe Thr Leu Thr Thr Ile Ala Val Asp Arg Tyr

145 150 155 160

Val Val Leu Val His Pro Leu Arg Arg Arg Ile Ser Leu Arg Leu Ser

165 170 175

Ala Tyr Ala Val Leu Ala Ile Trp Ala Leu Ser Ala Val Leu Ala Leu

180 185 190

Pro Ala Ala Val His Thr Tyr His Val Glu Leu Lys Pro His Asp Val

195 200 205

Arg Leu Cys Glu Glu Phe Trp Gly Ser Gln Glu Arg Gln Arg Gln Leu

210 215 220

Tyr Ala Trp Gly Leu Leu Leu Val Thr Tyr Leu Leu Pro Leu Leu Val

225 230 235 240

Ile Leu Leu Ser Tyr Val Arg Val Ser Val Lys Leu Arg Asn Arg Val

245 250 255

Val Pro Gly Cys Val Thr Gln Ser Gln Ala Asp Trp Asp Arg Ala Arg

260 265 270

Arg Arg Arg Thr Phe Cys Leu Leu Val Val Val Val Val Val Phe Ala

275 280 285

Val Cys Trp Leu Pro Leu His Val Phe Asn Leu Leu Arg Asp Leu Asp

290 295 300

Pro His Ala Ile Asp Pro Tyr Ala Phe Gly Leu Val Gln Leu Leu Cys

305 310 315 320
His Trp Leu Ala Met Ser Ser Ala Cys Tyr Asn Pro Phe Ile Tyr Ala
325 330 335
Trp Leu His Asp Ser Phe Arg Glu Glu Leu Arg Lys Leu Leu Val Ala
340 345 350
Trp Pro Arg Lys Ile Ala Pro His Gly Gln Asn Met Thr Val Ser Val
355 360 365
Val Ile
370

(2) INFORMATION FOR SEQ ID NO:22:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 206
(B) TYPE: Amino acid
(C) TOPOLOGY: Linear
(ii) MOLECULE TYPE: Peptide
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Leu Val Leu Val Ile Ala Arg Val Arg Arg Leu Tyr Asn Val Thr Asn
1 5 10 15
Phe Leu Ile Gly Asn Leu Ala Leu Ser Asp Val Leu Met Cys Thr Ala
20 25 30
Cys Val Pro Leu Thr Leu Ala Tyr Ala Phe Glu Pro Arg Gly Trp Val
35 40 45
Phe Gly Gly Gly Leu Cys His Leu Val Phe Phe Leu Gln Ala Val Thr
50 55 60
Val Tyr Val Ser Val Phe Thr Leu Thr Thr Ile Ala Val Asp Arg Tyr
65 70 75 80
Val Val Leu Val His Pro Leu Arg Arg Arg Ile Ser Leu Arg Leu Ser
85 90 95
Ala Tyr Ala Val Leu Ala Ile Trp Val Leu Ser Ala Val Leu Ala Leu
100 105 110
Pro Ala Ala Val His Thr Tyr His Val Glu Leu Lys Pro His Asp Val
115 120 125
Arg Leu Cys Glu Glu Phe Trp Gly Ser Gln Glu Arg Gln Arg Gln Leu
130 135 140

Tyr Ala Trp Gly Leu Leu Leu Val Thr Tyr Leu Leu Pro Leu Leu Val
145 150 155 160
Ile Leu Leu Ser Tyr Ala Arg Val Ser Val Lys Leu Arg Asn Arg Val
165 170 175
Val Pro Gly Arg Val Thr Gln Ser Gln Ala Asp Trp Asp Arg Ala Arg
180 185 190
Arg Arg Arg Thr Phe Cys Leu Leu Val Val Val Val Val Val
195 200 205

(2) INFORMATION FOR SEQ ID NO:23:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 126
(B) TYPE: Amino acid
(C) TOPOLOGY: Linear
(ii) MOLECULE TYPE: Peptide
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Val Val Leu Val His Pro Leu Arg Arg Arg Ile Ser Leu Arg Leu Ser
1 5 10 15
Ala Tyr Ala Val Leu Gly Ile Trp Ala Leu Ser Ala Val Leu Ala Leu
20 25 30
Pro Ala Ala Val His Thr Tyr His Val Glu Leu Lys Pro His Asp Val
35 40 45
Ser Leu Cys Glu Glu Phe Trp Gly Ser Gln Glu Arg Gln Arg Gln Ile
50 55 60
Tyr Ala Trp Gly Leu Leu Leu Gly Thr Tyr Leu Leu Pro Leu Leu Ala
65 70 75 80
Ile Leu Leu Ser Tyr Val Arg Val Ser Val Lys Leu Arg Asn Arg Val
85 90 95
Val Pro Gly Ser Val Thr Gln Ser Gln Ala Asp Trp Asp Arg Ala Arg
100 105 110
Arg Arg Arg Thr Phe Cys Leu Leu Val Val Val Val Val Val
115 120 125

(2) INFORMATION FOR SEQ ID NO:24:
(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 273
(B) TYPE: Nucleic acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: cDNA
(ix) FEATURE
(C) IDENTIFICATION METHOD: S
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

CTGGTGCTGG TGATCGCGCG GGTGCGCCGG CTGCACAACG TGACGAACTT CCTCATCGGC 60
AACCTGGCCT TGTCCGACGT GCTCATGTGC ACCGCCTGCG TGCCGCTCAC GCTGGCCTAT 120
GCCTTCGAGC CACGCGGCTG GGTGTTCGGC GGCGGCCTGT GCCACCTGGT CTTCTTCCTG 180
CAGCCGGTCA CCGTCTATGT GTCGGTGTTC ACGCTCACCA CCATCGCAGT GGACCGGTAC 240
GTCGTGCTGG TGCACCCGCT GAGGCGGCGC ATC 273

(2) INFORMATION FOR SEQ ID NO:25:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 177
(B) TYPE: Nucleic acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: cDNA
(ix) FEATURE
(C) IDENTIFICATION METHOD: S
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

GGCCTGCTGC TGGTCACCTA CCTGCTCCCT CTGCTGGTCA TCCTCCTGTC TTACGTCCGG 60
GTGTCAGTGA AGCTCCGCAA CCGCGTGGTG CCGGGCTGCG TGACCCAGAG CCAGGCCGAC 120
TGGGACCGCG CTCGGCGCCG GCGCACCTTC TGCTTGCTGG TGGTGGTCGT GGTGGTG 177

(2) INFORMATION FOR SEQ ID NO:26:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1110
(B) TYPE: Nucleic acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE

(C) IDENTIFICATION METHOD: S

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

ATGGCCTCAT CGACCACTCG GGGCCCCAGG GTTTCTGACT TATTTTCTGG GCTGCCGCCG 60
GCGGTCACAA CTCCCGCCAA CCAGAGCGCA GAGGCCTCGG CGGGCAACGG GTCGGTGGCT 120
GGCGCGGACG CTCCAGCCGT CACGCCCTTC CAGAGCCTGC AGCTGGTGCA TCAGCTGAAG 180
GGGCTGATCG TGCTGCTCTA CAGCGTCGTG GTGGTCGTGG GGCTGGTGGG CAACTGCCTG 240
CTGGTGCTGG TGATCGCGCG GGTGCGCCGG CTGCACAACG TGACGAACTT CCTCATCGGC 300
AACCTGGCCT TGTCCGACGT GCTCATGTGC ACCGCCTGCG TGCCGCTCAC GCTGGCCTAT 360
GCCTTCGAGC CACGCGGCTG GGTGTTCGGC GGCGGCCTGT GCCACCTGGT CTTCTTCCTG 420
CAGCCGGTCA CCGTCTATGT GTCGGTGTTC ACGCTCACCA CCATCGCAGT GGACCGCTAC 480
GTCGTGCTGG TGCACCCGCT GAGGCGGCGC ATCTCGCTGC GCCTCAGCGC CTACGCTGTG 540
CTGGCCATCT GGGCGCTGTC CGCGGTGCTG GCGCTGCCCG CCGCCGTGCA CACCTATCAC 600
GTGGAGCTCA AGCCGCACGA CGTGCGCCTC TGCGAGGAGT TCTGGGGCTC CCAGGAGCGC 660
CAGCGCCAGC TCTACGCCTG GGGGCTGCTG CTGGTCACCT ACCTGCTCCC TCTGCTGGTC 720
ATCCTCCTGT CTTACGTCCG GGTGTCAGTG AAGCTCCGCA ACCGCGTGGT GCCGGGCTGC 780
GTGACCCAGA GCCAGGCCGA CTGGGACCGC GCTCGGCGCC GGCGCACCTT CTGCTTGCTG 840
GTGGTGGTCG TGGTGGTGTT CGCCGTCTGC TGGCTGCCGC TGCACGTCTT CAACCTGCTG 900
CGGGACCTCG ACCCCCACGC CATCGACCCT TACGCCTTTG GGCTGGTGCA GCTGCTCTGC 960
CACTGGCTCG CCATGAGTTC GGCCTGCTAC AACCCCTTCA TCTACGCCTG GCTGCACGAC1020
AGCTTCCGCG AGGAGCTGCG CAAACTGTTG GTCGCTTGGC CCCGCAAGAT AGCCCCCCAT1080
GGCCAGAATA TGACCGTCAG CGTGGTCATC 1110

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 618

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Double

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE

(C) IDENTIFICATION METHOD: S

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

CTGGTGCTGG TGATCGCGCG GGTGCGCCGG CTGTACAACG TGACGAATTT CCTCATCGGC 60
AACCTGGCCT TGTCCGACGT GCTCATGTGC ACCGCCTGCG TGCCGCTCAC GCTGGCCTAT 120
GCCTTCGAGC CACGCGGCTG GGTGTTCGGC GGCGGCCTGT GCCACCTGGT CTTCTTCCTG 180
CAGGCGGTCA CCGTCTATGT GTCGGTGTTC ACGCTCACCA CCATCGCAGT GGACCGCTAC 240
GTCGTGCTGG TGCACCCGCT GAGGCGGCGC ATCTCGCTGC GCCTCAGCGC CTACGCTGTG 300
CTGGCCATCT GGGTGCTGTC CGCGGTGCTG GCGCTGCCCG CCGCCGTGCA CACCTATCAC 360
GTGGAGCTCA AGCCGCACGA CGTGCGCCTC TGCGAGGAGT TCTGGGGCTC CCAGGAGCGC 420
CAGCGCCAGC TCTACGCCTG GGGGCTGCTG CTGGTCACCT ACCTGCTCCC TCTGCTGGTC 480
ATCCTCCTGT CTTACGCCCG GGTGTCAGTG AAGCTCCGCA ACCGCGTGGT GCCGGGCCGC 540
GTGACCCAGA GCCAGGCCGA CTGGGACCGC GCTCGGCGCC GGCGCACCTT CTGCTTGCTG 600
GTGGTGGTCG TGGTGGTG 618

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 378

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Double

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE

(C) IDENTIFICATION METHOD: S

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

GTGGTTCTGG TGCACCCGCT ACGTCGGCGC ATTTCACTGA GGCTCAGCGC CTACGCGGTG 60
CTGGGCATCT GGGCTCTATC TGCAGTGCTG GCGCTGCCGG CCGCGGTGCA CACCTACCAT 120
GTGGAGCTCA AGCCCCACGA CGTGAGCCTC TGCGAGGAGT TCTGGGGCTC GCAGGAGCGC 180
CAACGCCAGA TCTACGCCTG GGGGCTGCTT CTGGGCACCT ATTTGCTCCC CCTGCTGGCC 240
ATCCTCCTGT CTTACGTACG GGTGTCAGTG AAGCTGAGGA ACCGCGTGGT GCCTGGCAGC 300
GTGACCCAGA GTCAAGCTGA CTGGGACCGA GCGCGTCGCC GCCGCACTTT CTGTCTGCTG 360
GTGGTGGTGG TGGTAGTG 378

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid
Synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

CGTGGSCMTS STGGGCAACN YCCTG 25

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid
Synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

GTNGWRRGGC ANCCAGCAGA KGGCAAA 27

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid
Synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

CTGTGYGYSA TYGCNNTKGA YMGSTAC 27

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid
Synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

AKGWAGWAGG GCAGCCAGCA GANSRYGAA 29

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid
Synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

CTGACTTATT TTCTGGGCTG CCGC 24

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid
Synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

AACACCGACA CATAGACGGT GACC 24

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid
Synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

GCICAYCARC AYTGYATGGA 20

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 26

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid
Synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

CCIACGGGIC KDATGCCICK GCCIGC 26

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 26

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid
Synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

ACGGGCCKDA TGCCICKGCC IGCRTA 26

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid
Synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

CCGGCGTACC AGGCAGGGTT 20

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid
Synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

AGGCAGGGTT GATGTCGGGG GTGCGGAT 28

(2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid
Synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

CTGCCAGCAG AGCCCACCAG CACTCCA 27

(2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid
Synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

GTGGGGGCCT GGCTCCTCTG CCTGCTG 27

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 32

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid
Synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

GTGTCGACGA ATGAAGGCGG TGGGGGCCTG GC 32

(2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid
Synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

AGGCTCCCGC TGTTATTCCT GGAC 24

(2) INFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 98

(B) TYPE: Amino acid

(C) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

Met Lys Ala Val Gly Ala Trp Leu Leu Cys Leu Leu Leu Leu Gly Leu
1 5 10 15
Ala Leu Gln Gly Ala Ala Ser Arg Ala His Gln His Ser Met Glu Ile
20 25 30
Arg Thr Pro Asp Ile Asn Pro Ala Trp Tyr Ala Gly Arg Gly Ile Arg
35 40 45
Pro Val Gly Arg Phe Gly Arg Arg Arg Ala Ala Leu Gly Asp Gly Pro
50 55 60
Arg Pro Gly Pro Arg Arg Val Pro Ala Cys Phe Arg Leu Glu Gly Gly
65 70 75 80
Ala Glu Pro Ser Arg Ala Leu Pro Gly Arg Leu Thr Ala Gln Leu Val
85 90 95
Gln Glu

(2) INFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 83

(B) TYPE: Amino acid

(C) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

Met Ala Leu Lys Thr Trp Leu Leu Cys Leu Leu Leu Leu Ser Leu Val
1 5 10 15
Leu Pro Gly Ala Ser Ser Arg Ala His Gln His Ser Met Glu Thr Arg
20 25 30
Thr Pro Asp Ile Asn Pro Ala Trp Tyr Thr Gly Arg Gly Ile Arg Pro
35 40 45
Val Gly Arg Phe Gly Arg Arg Arg Ala Thr Pro Arg Asp Val Thr Gly
50 55 60
Leu Gly Gln Leu Ser Cys Leu Pro Leu Asp Gly Arg Thr Lys Phe Ser
65 70 75 80
Gln Arg Gly

(2) INFORMATION FOR SEQ ID NO:46:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 249

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Double

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE

(C) IDENTIFICATION METHOD: S

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

ATGGCCCTGA AGACGTGGCT TCTGTGCTTG CTGCTGCTAA GCTTGGTCCT CCCAGGGGCT 60
TCCAGCCGAG CCCACCAGCA CTCCATGGAG ACAAGAACCC CTGATATCAA TCCTGCCTGG 120
TACACGGGCC GCGGGATCAG GCCTGTGGGC CGCTTCGGCA GGAGAAGGGC AACCCCGAGG 180
GATGTCACTG GACTTGGCCA ACTCAGCTGC CTCCCACTGG ATGGACGCAC CAAGTTCTCT 240
CAGCGTGGA 249

(2) INFORMATION FOR SEQ ID NO:47:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 31

(B) TYPE: Amino acid

(C) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

Ser Arg Ala His Gln His Ser Met Glu Thr Arg Thr Pro Asp Ile Asn
1 5 10 15
Pro Ala Trp Tyr Thr Gly Arg Gly Ile Arg Pro Val Gly Arg Phe
20 25 30

(2) INFORMATION FOR SEQ ID NO:48:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 32

(B) TYPE: Amino acid

(C) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

Ser Arg Ala His Gln His Ser Met Glu Thr Arg Thr Pro Asp Ile Asn
1 5 10 15
Pro Ala Trp Tyr Thr Gly Arg Gly Ile Arg Pro Val Gly Arg Phe Gly
20 25 30

(2) INFORMATION FOR SEQ ID NO:49:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 33

(B) TYPE: Amino acid

(C) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

Ser Arg Ala His Gln His Ser Met Glu Thr Arg Thr Pro Asp Ile Asn
1 5 10 15
Pro Ala Trp Tyr Thr Gly Arg Gly Ile Arg Pro Val Gly Arg Phe Gly
20 25 30
Arg

(2) INFORMATION FOR SEQ ID NO:50:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Amino acid

(C) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

Thr Pro Asp Ile Asn Pro Ala Trp Tyr Thr Gly Arg Gly Ile Arg Pro
1 5 10 15
Val Gly Arg Phe
20

(2) INFORMATION FOR SEQ ID NO:51:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21
(B) TYPE: Amino acid
(C) TOPOLOGY: Linear
(ii) MOLECULE TYPE: Peptide
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

Thr Pro Asp Ile Asn Pro Ala Trp Tyr Thr Gly Arg Gly Ile Arg Pro
1 5 10 15
Val Gly Arg Phe Gly
20

(2) INFORMATION FOR SEQ ID NO:52:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22
(B) TYPE: Amino acid
(C) TOPOLOGY: Linear
(ii) MOLECULE TYPE: Peptide
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:52:

Thr Pro Asp Ile Asn Pro Ala Trp Tyr Thr Gly Arg Gly Ile Arg Pro
1 5 10 15
Val Gly Arg Phe Gly Arg
20

(2) INFORMATION FOR SEQ ID NO:53:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 93
(B) TYPE: Nucleic acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: cDNA
(ix) FEATURE
(C) IDENTIFICATION METHOD: S
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:53:

AGCCGAGCCC ACCAGCACTC CATGGAGACA AGAACCCCTG ATATCAATCC TGCCTGGTAC 60
ACGGGCCGCG GGATCAGGCC TGTGGGCCGC TTC 93

(2) INFORMATION FOR SEQ ID NO:54:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 96
(B) TYPE: Nucleic acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: cDNA
(ix) FEATURE
(C) IDENTIFICATION METHOD: S
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:54:

AGCCGAGCCC ACCAGCACTC CATGGAGACA AGAACCCCTG ATATCAATCC TGCCTGGTAC 60
ACGGGCCGCG GGATCAGGCC TGTGGGCCGC TTCGGC 96

(2) INFORMATION FOR SEQ ID NO:55:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 99
(B) TYPE: Nucleic acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: cDNA
(ix) FEATURE
(C) IDENTIFICATION METHOD: S
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:55:

AGCCGAGCCC ACCAGCACTC CATGGAGACA AGAACCCCTG ATATCAATCC TGCCTGGTAC 60
ACGGGCCGCG GGATCAGGCC TGTGGGCCGC TTCGGCAGG 99

(2) INFORMATION FOR SEQ ID NO:56:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60
(B) TYPE: Nucleic acid
(C) STRANDEDNESS: Double

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE

(C) IDENTIFICATION METHOD: S

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:56:

ACCCCTGATA TCAATCCTGC CTGGTACACG GGCCGCGGGA TCAGGCCTGT GGGCCGCTTC 60

(2) INFORMATION FOR SEQ ID NO:57:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 63

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Double

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE

(C) IDENTIFICATION METHOD: S

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:57:

ACCCCTGATA TCAATCCTGC CTGGTACACG GGCCGCGGGA TCAGGCCTGT GGGCCGCTTC 60
GGC 63

(2) INFORMATION FOR SEQ ID NO:58:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 66

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Double

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE

(C) IDENTIFICATION METHOD: S

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:58:

ACCCCTGATA TCAATCCTGC CTGGTACACG GGCCGCGGGA TCAGGCCTGT GGGCCGCTTC 60
GGCAGG 66

(2) INFORMATION FOR SEQ ID NO:59:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 87

(B) TYPE: Amino acid

(C) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:59:

Met Lys Val Leu Arg Ala Trp Leu Leu Cys Leu Leu Met Leu Gly Leu
1 5 10 15

Ala Leu Arg Gly Ala Ala Ser Arg Thr His Arg His Ser Met Glu Ile
20 25 30

Arg Thr Pro Asp Ile Asn Pro Ala Trp Tyr Ala Ser Arg Gly Ile Arg
35 40 45

Pro Val Gly Arg Phe Gly Arg Arg Arg Ala Thr Leu Gly Asp Val Pro
50 55 60

Lys Pro Gly Leu Arg Pro Arg Leu Thr Cys Phe Pro Leu Glu Gly Gly
65 70 75 80

Ala Met Ser Ser Gln Asp Gly
85

(2) INFORMATION FOR SEQ ID NO:60:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 261

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Double

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE

(C) IDENTIFICATION METHOD: S

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:60:

ATGAAGGTGC TGAGGGCCTG GCTCCTGTGC CTGCTGATGC TGGGCCTGGC CCTGCGGGGA 60

GCTGCAAGTC GTACCCATCG GCACTCCATG GAGATCCGCA CCCCTGACAT CAATCCTGCC 120

TGGTACGCCA GTCGCGGGAT CAGGCCTGTG GGCCGCTTCG GTCGGAGGAG GCAACCCTG 180

GGGGACGTCC CCAAGCCTGG CCTGCGACCC CGGCTGACCT GCTTCCCCCT GGAAGGCGGT 240

GCTATGTCGT CCCAGGATGG C 261

(2) INFORMATION FOR SEQ ID NO:61:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 31

(B) TYPE: Amino acid

(C) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:61:

Ser Arg Thr His Arg His Ser Met Glu Ile Arg Thr Pro Asp Ile Asn
1 5 10 15

Pro Ala Trp Tyr Ala Ser Arg Gly Ile Arg Pro Val Gly Arg Phe
20 25 30

(2) INFORMATION FOR SEQ ID NO:62:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 32

(B) TYPE: Amino acid

(C) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:62:

Ser Arg Thr His Arg His Ser Met Glu Ile Arg Thr Pro Asp Ile Asn
1 5 10 15

Pro Ala Trp Tyr Ala Ser Arg Gly Ile Arg Pro Val Gly Arg Phe Gly
20 25 30

(2) INFORMATION FOR SEQ ID NO:63:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 33

(B) TYPE: Amino acid

(C) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:63:

Ser Arg Thr His Arg His Ser Met Glu Ile Arg Thr Pro Asp Ile Asn
1 5 10 15
Pro Ala Trp Tyr Ala Ser Arg Gly Ile Arg Pro Val Gly Arg Phe Gly
20 25 30
Arg

(2) INFORMATION FOR SEQ ID NO:64:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20
(B) TYPE: Amino acid
(C) TOPOLOGY: Linear
(ii) MOLECULE TYPE: Peptide
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:64:

Thr Pro Asp Ile Asn Pro Ala Trp Tyr Ala Ser Arg Gly Ile Arg Pro
1 5 10 15
Val Gly Arg Phe
20

(2) INFORMATION FOR SEQ ID NO:65:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21
(B) TYPE: Amino acid
(C) TOPOLOGY: Linear
(ii) MOLECULE TYPE: Peptide
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:65:

Thr Pro Asp Ile Asn Pro Ala Trp Tyr Ala Ser Arg Gly Ile Arg Pro
1 5 10 15
Val Gly Arg Phe Gly
20

(2) INFORMATION FOR SEQ ID NO:66:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22
(B) TYPE: Amino acid

(C) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:66:

Thr Pro Asp Ile Asn Pro Ala Trp Tyr Ala Ser Arg Gly Ile Arg Pro
1 5 10 15

Val Gly Arg Phe Gly Arg
20

(2) INFORMATION FOR SEQ ID NO:67:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 93

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Double

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE

(C) IDENTIFICATION METHOD: S

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:67:

AGTCGTACCC ATCGGCACTC CATGGAGATC CGCACCCCTG ACATCAATCC TGCCTGGTAC 60
GCCAGTCGCG GGATCAGGCC TGTGGGCCGC TTC 93

(2) INFORMATION FOR SEQ ID NO:68:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 96

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Double

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE

(C) IDENTIFICATION METHOD: S

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:68:

AGTCGTACCC ATCGGCACTC CATGGAGATC CGCACCCCTG ACATCAATCC TGCCTGGTAC 60
GCCAGTCGCG GGATCAGGCC TGTGGGCCGC TTCGGT 96

(2) INFORMATION FOR SEQ ID NO:69:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 99

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Double

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE

(C) IDENTIFICATION METHOD: S

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:69:

AGTCGTACCC ATCGGCACTC CATGGAGATC CGCACCCCTG ACATCAATCC TGCCTGGTAC 60

GCCAGTCGCG GGATCAGGCC TGTGGGCCGC TTCGGTCGG 99

(2) INFORMATION FOR SEQ ID NO:70:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 60

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Double

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE

(C) IDENTIFICATION METHOD: S

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:70:

ACCCCTGACA TCAATCCTGC CTGGTACGCC AGTCGCGGGA TCAGGCCTGT GGGCCGCTTC 60

(2) INFORMATION FOR SEQ ID NO:71:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 63

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Double

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE

(C) IDENTIFICATION METHOD: S

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:71:

ACCCCTGACA TCAATCCTGC CTGGTACGCC AGTCGCGGGA TCAGGCCTGT GGGCCGCTTC 60
GGT 63

(2) INFORMATION FOR SEQ ID NO:72:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 66

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Double

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE

(C) IDENTIFICATION METHOD: S

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:72:

ACCCCTGACA TCAATCCTGC CTGGTACGCC AGTCGCGGGA TCAGGCCTGT GGGCCGCTTC 60
GGTCGG 66

(2) INFORMATION FOR SEQ ID NO:73:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21

(B) TYPE: Amino acid

(C) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(ix) FEATURE: Xaa of the 10th position is Ala or Thr.
Xaa of the 11th position is Gly or Ser.
Xaa of the 21st position is H, Gly or
GlyArg.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:73:

Thr Pro Asp Ile Asn Pro Ala Trp Tyr Xaa Xaa Arg Gly Ile Arg Pro
1 5 10 15

Val Gly Arg Phe Xaa
20

(2) INFORMATION FOR SEQ ID NO:74:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 11

(B) TYPE: Amino acid

(C) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(ix) FEATURE: Xaa of the 3rd position is Ala or Thr.
Xaa of the 5th position is Gln or Arg.
Xaa of the 10th position is Ile or Thr.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:74:

Ser Arg Xaa His Xaa His Ser Met Glu Xaa Arg
1 5 10

(2) INFORMATION FOR SEQ ID NO:75:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 26

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid
Synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:75:

CARCAYTCCA TGGAGACAAG AACCCC 26

(2) INFORMATION FOR SEQ ID NO:76:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid
Synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:76:

TACCAGGCAG GATTGATACA GGGG 24

(2) INFORMATION FOR SEQ ID NO:77:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid
Synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:77:

GGCATCATCC AGGAAGACGG AGCAT 25

(2) INFORMATION FOR SEQ ID NO:78:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid
Synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:78:

AGCAGAGGAG AGGGAGGGTA GAGGA 25

(2) INFORMATION FOR SEQ ID NO:79:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid
Synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:79:

ACGTGGCTTC TGTGCTTGCT GC 22

(2) INFORMATION FOR SEQ ID NO:80:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid
Synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:80:

GCCTGATCCC GCGGCCCGTG TACCA 25

(2) INFORMATION FOR SEQ ID NO:81:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 26

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid
Synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:81:

TTGCCCTTCT CCTGCCGAAG CGGCCC 26

(2) INFORMATION FOR SEQ ID NO:82:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid
Synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:82:

GGCGGGGGCT GCAAGTCGTA CCCATCG 27

(2) INFORMATION FOR SEQ ID NO:83:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid
Synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:83:

CGGCACTCCA TGGAGATCCG CACCCCT 27

(2) INFORMATION FOR SEQ ID NO:84:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid
Synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:84:

CAGGCAGGAT TGATGTCAGG GGTGCGG 27

(2) INFORMATION FOR SEQ ID NO:85:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid
Synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:85:

CATGGAGTGC CGATGGGTAC GACTTGC 27

(2) INFORMATION FOR SEQ ID NO:86:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid
Synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:86:

GGCCTCCTCG GAGGAGCCAA GGGATGA 27

(2) INFORMATION FOR SEQ ID NO:87:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid
Synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:87:

GGGAAAGGAG CCCGAAGGAG AGGAGAG 27

(2) INFORMATION FOR SEQ ID NO:88:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid
Synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:88:

CCTGCTGGCC ATTCTCCTGT CTTAC 25

(2) INFORMATION FOR SEQ ID NO:89:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid
Synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:89:

GGGTCCAGGT CCCGCAGAAG GTTGA 25

(2) INFORMATION FOR SEQ ID NO:90:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid
Synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:90:

GAAGACGGAG CATGGCCCTG AAGAC 25

(2) INFORMATION FOR SEQ ID NO:91:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid
Synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:91:

GGCAGCTGAG TTGGCCAAGT CCAGT 25

(2) INFORMATION FOR SEQ ID NO:92:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15

(B) TYPE: Amino acid

(C) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:92:

Ser Arg Ala His Gln His Ser Met Glu Ile Arg Thr Pro Asp Cys
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:93:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15

(B) TYPE: Amino acid

(C) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:93:

Cys Ala Trp Tyr Ala Gly Arg Gly Ile Arg Pro Val Gly Arg Phe
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:94:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15

(B) TYPE: Amino acid

(C) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:94:

Cys Glu Ile Arg Thr Pro Asp Ile Asn Pro Ala Trp Tyr Ala Gly
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:95:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid
Synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:95:

AGATTGGCAT CATCCAGGAA GACGGAGCAT 30

(2) INFORMATION FOR SEQ ID NO:96:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 31

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid
Synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:96:

GCTGACTCGA CAGCACTGTC TTCTCGAGCT G 31

(2) INFORMATION FOR SEQ ID NO:97:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid
Synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:97:

AACCCCTTCA TCTATGCGTG G 21

(2) INFORMATION FOR SEQ ID NO:98:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid
Synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:98:

ATATTCTGGC CATGAGGCAC 20

(2) INFORMATION FOR SEQ ID NO:99:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28

(B) TYPE: Nucleic acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid
Synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:99:

TTCCGAGAGG AGCTACGCAA GATGCTTC 28